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THE MECHANISM FOR SYNCHRONOUS SPAWNING IN HYDRACTINIA AND PENNARIA

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Introduction

Synchronous spawning of all individuals in one neighborhood at a particular time of day occurs in various Hydrozoa. It has been noted in *Pennaria* and *Clava* by Beckwith (1909), in *Halyclistis* and *Sertularia* by the Teissiers (1927), and in *Hydractinia* by various workers. These are all colonial species, and the sexual individuals are sessile. Moreover, only one sex is normally represented in each colony, and male and female colonies may live at some distance from each other. If all the eggs and sperm are discharged at the same moment in a given mud flat or tide pool, the chance of survival of the genus is greatly enhanced. How this happens is the subject of the present study.

There have been conflicting reports as to the regular spawning time of *Hydractinia*. Bunting (1894) observed it to be between 9.30 and 10.30 P.M., Teissier and Teissier (1927) between 7 and 8 A.M. C. W. Hargitt (1911) found spawning to occur at various times of the day but most frequently in the early morning. My own observations showed that *Hydractinia* in a state of nature always spawns shortly after sunrise. All but the Teissiers worked at Woods Hole.

Reports from many observers are unanimous that *Pennaria* sheds its eggs and sperm normally several hours after sunset (G. T. Hargitt, 1900; Baker, 1936).

Attempts to force both these species to spawn at any desired hour of day or night have now proved successful, and have opened an approach to the problem of the nature of the spawning mechanism. Reasons can be offered for the conflicting reports on *Hydractinia* and for the difference in preferred spawning time between *Hydractinia* and *Pennaria*.

Methods of Controlling Spawning

Hydractinia echinata Fleming is in good breeding condition at Woods Hole in June and July. The colonies, growing on shells inhabited by

hermit crabs, may be kept in open dishes of sea water and will shed eggs or sperm copiously once a day for a week before becoming exhausted.

It was first noted that *Hydractinia*'s sunrise spawning could be delayed indefinitely by leaving the colonies in the dark of a desk drawer or loose box. When they were returned to the light they spawned in about an hour, with some variation in time according to the condition of the colonies.

Colonies continuously illuminated during the night from an ordinary reading lamp omitted the normal spawning at sunrise, and would not spawn at any later time until they had first been darkened for a while and then restored to light.

In practice, spawning was produced in *Hydractinia* at will by (1) leaving the colonies in running sea water under a glowing 100-watt light bulb from the time of collection until needed; (2) then placing them in the dark for from one to several hours; (3) then exposing them to daylight or a desk lamp, at which time the colonies could be examined with a hand lens and segregated according to sex. Practically all male colonies in prime condition would spawn 50 minutes after the moment of re-illumination (Fig. 1), female colonies five minutes later (Fig. 2).

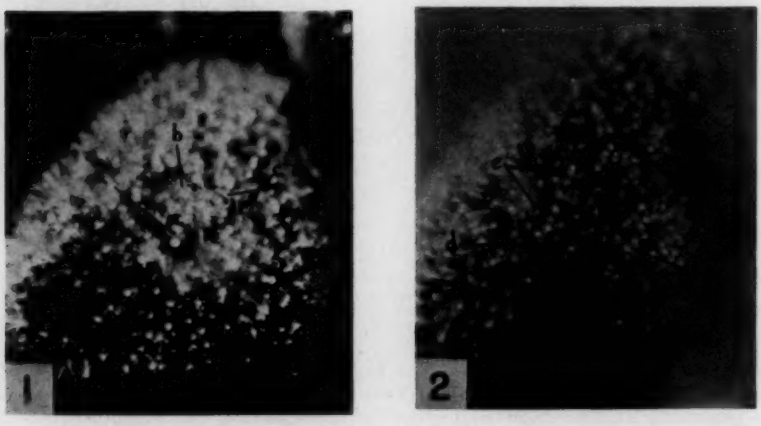
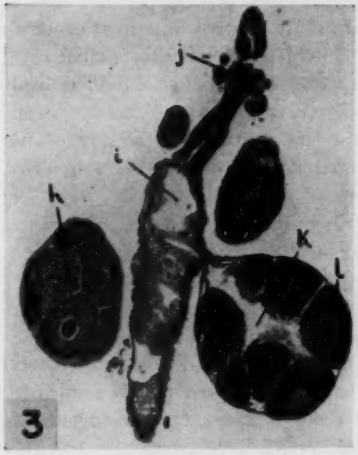
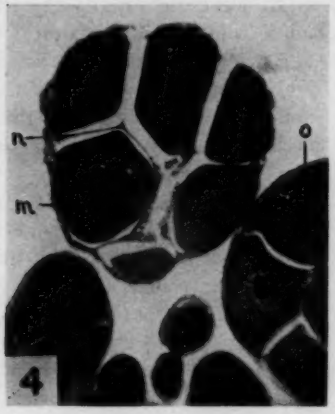


PLATE I

FIG. 1. Male colony of *Hydractinia* just starting to shed sperm. (*a*) Gonozoöid bearing two white ripe gonophores and two translucent unripe gonophores; (*b*) feeding polyp; (*c*) spiral streams of sperm from ruptured gonophores. Toward lower right, a foggy area full of dispersing sperm. About 6 ×.

FIG. 2. Female colony of *Hydractinia* just starting to shed eggs. (*d*) Gonophores containing ripe unshed eggs; (*e*) feeding polyps; (*f*) clusters of eggs just shed; (*g*) stinging polyps around lip of snail shell. About 6 ×.





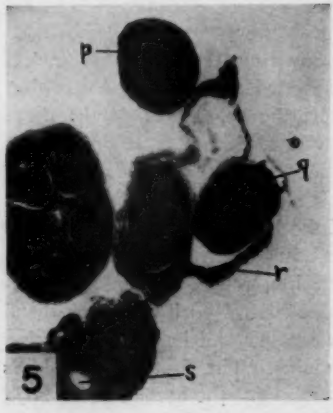


PLATE II

FIG. 3. Section of *Hydractinia* gonozoöid 45 minutes before spawning. (*h*) Gonophore containing unripe eggs; (*i*) gastrovascular cavity of gonozoöid; (*j*) distal end of gonozoöid with nematocysts; (*k*) gastrodermal spadix of ripening gonophore; (*l*) peripherally placed enlarged germinal vesicle of ripening egg. About 60 ×.

FIG. 4. Section of *Hydractinia* female gonophore just before shedding. (*m*) Mature egg cell; (*n*) egg follicle; (*o*) immature oöcyte in a neighboring gonophore. About 100 ×.

FIG. 5. Section of *Hydractinia* female gonophore preserved during the act of spawning. (*p*) Discharged egg; (*q*) egg emerging; (*r*) collapsing wall of gonophore, showing outer epidermis and inner musculo-epithelium; (*s*) gastrovascular cavity of parent gonozoöid. About 100 ×.

FIG. 6. Section showing three *Hydractinia* eggs in late cleavage stages still within the gonophore. Maturation induced by dark and light treatment, shedding inhibited by calcium-free sea water. About 100 ×.

In *Pennaria tiarella* Ayres, both uninterrupted illumination and uninterrupted darkness were likewise found to inhibit the normal evening spawning, as had earlier been suggested by Baker (1936). But after a prolonged period in the dark, spawning took place 10 to 14 hours after return to the light, irrespective of the time of day. The mechanism seems similar, but *Pennaria* reacts far more slowly to light than *Hydractinia*.

Since laboratory aquaria may be in quite dark corners, and since the turning on of lights in the laboratory may precipitate the shedding reactions, it is not surprising that earlier investigators should have reported synchronous spawning of *Hydractinia* at various times of the day. The suggestion of Bunting (1894) that heat and cold control the spawning of *Hydractinia* has not been confirmed, and her experiments in this direction are invalidated by the fact that storing the colonies in a (dark) icebox and later removing them to room temperature (and light) will produce the same result whether the icebox be warm or cold.

Maturation Phenomena

Early accounts of maturation in *Hydractinia* eggs were incomplete or fallacious because material was not collected at the right time of day. Beckwith (1914) was the first to report the cytological details, from study of a quantity of material preserved at dawn. The present investigation corroborates her findings.

Whether spawning be the normal sunrise event or whether it be produced at another time by control of the lighting, the hour following re-illumination and prior to shedding is occupied in maturation. Since the preceding spawning period, the germinal vesicle has enlarged, become sharply granular and moved from a central position to the distal cell wall (Fig. 3), and it now becomes smaller and hazy, disappearing about half an hour after the light stimulus. The first polar body is given off at the same distal point in 45 minutes, the second polar body ten minutes later. The first polar body often divides. At this time, 55 minutes from the time of re-illumination in most material, the wall of the gonophore ruptures (Fig. 4) and the eggs come oozing out (Fig. 5).

Each new egg is covered by a highly transparent but radially striated jelly, which has not been mentioned by previous workers. The jelly swells considerably on contact with sea water, pulling the polar bodies away from the egg surface and pushing the egg away from the collapsing gonophore. Very soon the polar bodies are so far from the egg that they are brushed off by the least disturbance, and lost.

Dissection experiments show that the process of maturation is initiated solely by the action of light on the oöcyte itself, and not by any

general action on the colony or the individual. Single gonozoöids isolated from either male or female colonies lived well for five days in watch glasses of sea water, and responded either to the succession of night and day by normal shedding at sunrise, or to controlled illumination by shedding when experimentally planned. Single gonophores isolated from the gonozoöids did likewise, although it was not possible to separate in advance those which would shed from those that would not. In practice, a batch was made up by collecting the ripest-looking single gonophore from each of a number of very well developed gonozoöids; depending on the actual ripeness of the parent colony, anywhere from 0 to 100 per cent of the gonophores so selected would shed their eggs after dark-light treatment.

When large numbers of apparently ripe primary oöcytes were dissected out of gonophores selected in this fashion, and these isolated oöcytes were then subjected to the routine of one hour of darkness and one hour of light, some of them could be watched passing through maturation stages according to the expected timetable, and could later undergo fertilization and cleavage. Sperm added before or during maturation were without effect until emergence of the second polar bodies. The proportion of maturing to non-maturing eggs in a large group of these dissected eggs was the same as the proportion in the control group of intact gonophores from the same colony. In other words, light could stimulate maturation in the isolated oöcyte with the same facility that it did in the normal animal.

Preparation by Darkness

The amount of time required in the dark to prepare the *Hydractinia* colonies for shedding is certainly short, although no spawning will take place in continuously illuminated colonies. Eggs can be ripe enough so that only 15 minutes or less in the dark will prepare them for reaction to light, but a longer period is necessary for maximum yield of eggs. In a group of 36 female colonies that were allowed just 15 minutes in the dark, only one shed any eggs after re-illumination. Eighteen of these same colonies were given several more hours in the dark, and all shed heavily following the second re-illumination.

Performance of colonies that were darkened for half an hour was somewhat better. Forty-two female colonies were kept illuminated for 36 hours or more, then darkened for half an hour. Only 12 shed eggs after re-illumination. Darkness for an hour or longer followed by re-illumination usually produces spawning in 100 per cent of prime fresh colonies.

What takes place within the eggs during the dark period is a question. No nuclear changes are discernible in fresh or sectioned material. Until some time after re-illumination, it is not possible to distinguish the eggs that are going to be shed from the large unripe eggs in neighboring gonophores. Perhaps darkness allows the accumulation of light-sensitive substances whose breakdown initiates the maturation processes.

Spawning after Re-illumination

In a few cases, colonies kept in a box which admitted a crack of light on one side were found to have spawned before return to full daylight, but when colonies were put in complete photographic darkness after prolonged illumination, eggs were never shed in the dark. Ten female colonies were kept in complete darkness for 28½ hours, without shedding a single egg. On removal to light, all ten colonies shed within the hour.

To test the effectiveness of a short dosage of light, 76 female colonies which had been continuously illuminated for 24 hours since they had been collected were divided into two apparently equivalent batches and stored in complete darkness for 4¼ hours. One was to shed after a brief exposure to light, the other after prolonged exposure to light, and the relative effectiveness of the exposures was to be tested by capacity for re-spawning after a second immediately succeeding period of darkness.

Batch A was illuminated for 80 seconds at 1.15 P.M. and restored to the dark. At 3.15 P.M. it was taken from the dark and the heavy spawn of eggs was removed. Left on the desk in daylight, this batch was then expected to shed more eggs in one hour, but only an insignificant several hundred eggs were produced by 5 P.M.

Batch B was left on the desk in full north window daylight from 1.15 to 2.30 P.M. Heavy shedding of eggs took place from 2.10 to 2.15 P.M., and these eggs were removed. From 2.30 until 3.45 P.M. batch B was stored in complete darkness and then placed in the window by the side of batch A. Only a few dozen eggs were produced in the second spawning before 5 P.M.

Batches A and B were kept illuminated overnight and the experiment was repeated the next day. This time batch A was given only 12 seconds of light at 1.15 P.M. Batch B stayed in the light an hour and a quarter. Both batches shed heavily, in approximately equal quantity. As before, the total elapsed time from first darkness to last illumination was the same in the two batches. After the second dark period, batch A shed only several hundred eggs, batch B shed none at all.

Eggs from each different batch were funneled into a standing column in a burette to measure their volume, and a rough estimate of their number was calculated from counts of samples of known volume. Each batch produced well over 6,000 eggs on each day. Batch B was slightly more productive than batch A in the sum of its two sheddings the first day, but there was no significant difference the second day.

It is clear that from 12 to 80 seconds of strong daylight is sufficient stimulus to bring about a heavy shedding of dark-prepared eggs, and that illumination in excess of this is able to bring about the discharge of only a scattering few more eggs. It is not at all essential that the colonies remain in the light during the period from their re-illumination until the time they are shed.

However, a very brief period of illumination depends for its effectiveness on an ample period of previous darkness. On July 18, 50 female colonies which had been continuously illuminated for over 24 hours were stored in the dark for one hour, exposed then to ten seconds of light from a 100-watt bulb two feet away, and then kept in the dark for 2¾ hours. No eggs whatever were shed, but after standing for an hour longer under the light bulb, a heavy spawn resulted. On the following day, 34 of these same colonies, after 13 hours of illumination, were given an hour in the dark, ten seconds of daylight (north window, midday), and 1½ hours in the dark. On removal to daylight again, a moderate shedding of eggs was found, but within the hour twice as many more eggs were shed as a result of the new illumination. No explanation can be offered why a procedure closely similar to that which failed to cause shedding the first day should succeed the following day. But in both cases it is clear that ten seconds of light, which would have been sufficient to precipitate a near-maximum shedding if the colonies had been kept a long time in the dark, was far less effective after only one hour's darkness.

Role of the Jelly Layer in Egg Shedding

Since the eggs are obviously under considerable pressure inside the gonophore during their maturation phase, and since their jelly layer expands while they ooze outward, it was considered whether the jelly was not the immediate agent in rupturing the gonophore and inducing shedding, simply by the absorption of water. Several considerations make this appear unlikely.

Immature eggs are coated with a dense but entirely transparent layer of jelly tightly confined inside a very thin sphere of flattened follicle cells (Fig. 4). When oöcytes with large germinal vesicles are dissected free from the gonophore, the rupture of the follicle-cell layer causes the jelly

to swell rapidly and it begins to show very delicate radial striations. If the follicle-cell sphere remains unbroken, the jelly remains unswollen for an indefinite period. This indicates that if the swelling of the jelly causes shedding of the egg there must exist also some egg-induced rupture or altered permeability in the follicle-cell layer, if not the gonophore wall also, allowing the jelly to imbibe water.

But there is evidence that eggs can be shed from the gonophore after destruction of the jelly. Of several treatments which resulted in the dispersal of jelly, the one least harmful to eggs or polyps was immersion in calcium-free sea water. *Hydractinia* can survive without permanent ill effects in Van't Hoff's artificial sea water with $CaCl_2$ omitted for upwards of 14 hours, *Pennaria* for a considerably shorter period.

Hydractinia gonophores containing eggs undergoing maturation, and gonophores containing germinal-vesicle stages, were rinsed in this solution, and the eggs dissected out in it. Some eggs showed very thin and watery jelly, but most showed none at all. *Pennaria* eggs similarly dissected from the medusae in this solution were invariably obtained without jelly, whereas those dissected out in normal sea water had a thick jelly coat best seen in ink suspension. This effect having been observed, efforts were made in *Hydractinia* to disperse the jelly before shedding could start.

Ten female colonies were kept in the dark nine hours and brought to the light, being transferred at once to calcium-free sea water in which they remained one hour. Returned then to normal sea water, they recovered enough to produce a heavy spawn of eggs in another hour and a half, but practically none of these eggs showed any jelly at all. They could be packed together in contact with each other, which cannot be done with ordinary eggs unless the jelly is removed by violent shaking. These jelly-less eggs were mature, and when inseminated they cleaved on schedule.

Calcium Essential for Spawning

If male or female colonies of *Hydractinia* are exposed to the action of calcium-free sea water for three quarters of an hour or longer just prior to the expected shedding time, no spawning occurs in the solution, and a recovery period is necessary in normal sea water before eggs or sperm are released. The corresponding reactions in the more sensitive *Pennaria* are striking.

Pennaria has well-developed medusa-gonophores, in contrast to the degenerate sporosac-gonophores of *Hydractinia*. When *Pennaria* is

ready to spawn, the pendant medusae open their bells, and the inner musculo-epithelium of each starts a rhythmic pulsation which is so prolonged and powerful as to accomplish the discharge of the sexual products. When a colony containing many pulsating medusae is transferred to calcium-free sea water, the musculo-epithelium of each medusa is almost instantly paralyzed. Not even the slightest muscular tremor is found after one minute in the solution. Transfer back to normal sea water is followed by prompt recovery, and at the end of a minute or two the medusae are pulsating as strongly as before.

In *Hydractinia,* the inner musculo-epithelium shares in the degenerate development of the whole sporosac (Goette, 1907, Plates V, VI), and is difficult to demonstrate except during the actual process of extrusion of the eggs (Fig. 5). This and the fact that it is not directly exposed to the action of the fluid until the moment of spawning in the sporosac may explain why *Hydractinia* does not show the extreme rapidity of response to lack of calcium exhibited by *Pennaria.*

Since the calcium-free sea water has been shown to have an effect on the jelly layer around the egg, the possibility of inhibition of shedding also through direct injury to the egg must be considered.

Calcium-free sea water apparently does not hinder the darkness-preparation of the oöcytes inside the gonophores. One colony, remaining in this solution 1½ hours in the dark, was transferred to normal sea water in the light, and produced eggs in 65 minutes. Another colony, after one hour in the dark in this solution, was illuminated then for one minute during transfer to normal sea water and darkness, and presently shed eggs in the dark.

The question whether calcium is necessary for the carrying out of the maturation processes and the initiation of them by light has not been put to critical test. However, as proof that the dosage of calcium-free sea water can be adjusted so as to allow successful maturation and yet inhibit shedding, a case of experimentally produced internal fertilization in *Hydractinia* may be cited.

Twelve female colonies were put into calcium-free sea water in the dark for one hour. Next they spent 30 minutes in the light in normal sea water, then an hour in the light in calcium-free sea water again. No eggs having been shed at the end of that time, the gonophores were carefully scrutinized under the microscope with strong light, and the absence of germinal vesicles noted in a number of the eggs. The colonies were left in normal sea water from then on, and a few eggs were extruded in the next two hours. Examination still showing unshed matured eggs in some of the gonophores, sperm were added to the

dishes. Figure 6 shows a section through a gonophore which contained three unshed eggs in late cleavage stages, preserved three and a half hours later.

In many hydrozoan genera, such internal development of embryos is normal. Nothing like it ever occurs normally in *Hydractinia*.

Discussion

Influence of light on breeding seasons or spawning reactions is well known in a number of phyla. In most animals the germ cells are well shielded from light and the action is indirect, through the nervous system or in more subtle ways. Even in the translucent ascidians whose spawning is controllable by light (Rose, 1939), some fertilizable eggs can be obtained by dissection at any time of day or night. I cannot cite any other case in which light directly initiates the maturation processes of animals.

One not altogether consistent case among the Chlorophyceae deserves mention. Kater (1929) found that *Chlamydomonas nasuta* underwent cell division regularly and almost exclusively between 9 and 12 P.M. On the other hand Moewus (1933) reports that *Chlamydomonas eugametos* undergoes cell division both in light and dark, but more cell division occurs in regular 12-hour alternations of light and dark than in light. If the cells, lighted for 12 hours, are then kept dark more than 48 hours, all cell divisions cease, even with all other conditions favoring.

Moewus presents some rather remarkable observations on light-controlled sexual activity in *Chlamydomonas*. The gametes never copulate in the dark. When brought into the light, they must be exposed for 30 to 45 minutes before they are ready to copulate. This delay, perhaps representing some sort of physiological maturation, could be reduced by soaking the unlighted gametes in water which had stood for some time in the light with other gametes of the same sexual strain in it, and then been filtered free of them. According to Moewus the soluble effective substances produced by the gametes in the light are highly sex-specific, and those of opposed sex neutralize each other. Other genera of green algae studied by Moewus revealed none of these peculiar reactions to light.

In *Hydractinia* the fact that shedding of eggs occurs only in a few of the large numbers of gonophores in a colony, and the fact that only mature eggs are shed, suggests that the actual shedding process is initiated by some late-maturation change in the eggs themselves. Whether this is a mere increase in volume which ruptures the gonophores, or a chemical stimulation of the musculo-epithelium, or what, is the subject of further experiments now in progress.

Summary

1. Methods are reported for scheduling the shedding of eggs and sperm by *Hydractinia* and *Pennaria* at any desired time of day.

2. Maturation in these animals occurs only after the germ cells have undergone a period of preparation in the darkness, and is initiated by direct action of light on the germ cells. Once initiated by light, maturation can proceed in the dark. Uninterrupted light, or uninterrupted darkness, results in complete cessation of maturation and spawning.

3. In *Hydractinia* maximum shedding requires at least an hour's darkness-preparation, although less time is sufficient for some eggs. Ten seconds of illumination is sufficient for widespread but not maximum initiation of maturation.

4. The hypothesis is advanced that darkness allows the accumulation of light-sensitive substances within the germ cell, whose breakdown initiates the process of maturation. Oöcytes dissected free from the organism can be matured by appropriate dark-light treatment.

5. Shedding of eggs and sperm takes place simultaneously with the completion of the maturation process. The swelling of jelly around the egg is not an important factor in bringing about shedding. Evidence is presented that contraction of the gonophore wall is an important factor.

6. Paralysis of the gonophore's musculo-epithelium by calcium-free sea water inhibits the shedding of mature eggs and sperm. Fertilization and cleavage of eggs within the gonophore have been produced in *Hydractinia* by causing maturation, inhibiting shedding and adding sperm.

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A QUANTITATIVE STUDY OF THE INTERRELATIONSHIP OF OXYGEN AND HYDROGEN ION CONCENTRATION IN INFLUENCING TUBULARIA REGENERATION

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Increased hydrogen ion concentration of sea water has an inhibitory effect on *Tubularia* regeneration (Komori, 1933; Miller, 1939; Goldin, 1942). Goldin presented evidence supporting the view that the effectiveness of oxygen in regeneration is limited by the inhibitory action of accumulated acid metabolites. In addition, experiments in which the hydrogen ion concentration of the sea water was increased, while the oxygen tension was maintained at a constant level, showed that there is a pH range in which regeneration is retarded, and a critical pH below which regeneration will not occur. It was also indicated that the critical pH is related to the oxygen tension.

Thus the ability to form primordia appears to be dependent upon the hydrogen ion concentration as well as the oxygen tension (Barth, 1938a, 1940) of the sea water. These experiments were designed in order to study the interrelationship of these factors in their effects on *Tubularia* regeneration. The author wishes to thank Professors L. G. Barth and H. B. Steinbach for their guidance during the course of these investigations.

Methods

The general procedure for selecting the *Tubularia* stems, treating them and measuring rate of regeneration, was essentially the same as that described by Barth (1938a, 1938b). Young, unbranched stems of uniform diameter were selected from young colonies. Stem segments 6 mm. in length were used for the experiments, the required length being removed from the region 5 to 11 mm. proximal to the hydranth. In order to insure uniformity of material, random samples of these stem segments were taken for each experiment.

The oxygen tension and the hydrogen ion concentration of the sea water were adjusted by saturating the sea water with oxygen-carbon

dioxide-nitrogen gas mixtures. The gases were drawn from cylinders and the mixtures were prepared in a graduated 20 gallon carboy by the standard method of water displacement. The carboy was filled with water, and the water was then displaced by the required quantity of each gas. Adjustment to atmospheric pressure was made after the addition of each gas, so that the total pressure was one atmosphere. The nitrogen gas was used in order to bring all of the mixtures to the same total volume. For each experiment a series of gas mixtures was prepared containing carbon dioxide at increasing partial pressures and oxygen at the same partial pressure. Measured samples of filtered sea water (350 ml.) were introduced into one liter florence flasks and then saturated with the required gas mixtures. In this way, for each experiment, the hydrogen ion concentration (carbon dioxide tension) of the sea water was varied, while the oxygen tension was maintained at a constant level. The various experiments, however, were conducted at different levels of oxygen tension, so that a wide range of oxygen and hydrogen ion concentration was covered. Twenty stem segments were introduced into each flask, and samples of sea water were removed in order to determine the oxygen tension and the pH. Dissolved oxygen was determined by the Winkler method, and the hydrogen ion concentration was measured with a Beckman pH meter. The experimental flasks were kept at constant temperature ($19 \pm 1°$ C.) during the above operations and throughout the course of the experiments.

The stems were then examined for regeneration. They were removed from the flasks as the primordia were formed, by means of a long pipette. The time was noted, and the primordia were measured with the aid of an ocular micrometer mounted in a binocular microscope. The formula $R = L/t =$ R. U. (regenerative units) was used for measuring rate of regeneration, where L is the length of the primordium in micra, and t is the time in hours at which the primordium becomes constricted from the stem segment proper. At low oxygen and high hydrogen ion concentration many of the stems did not regenerate. Stems which failed to regenerate were removed from the flasks at the close of the experiment (106–120 hours), and placed in running sea water. The stems which regenerated after removal were considered to be inhibited, and the length of their primordia as well as their rate of regeneration were considered as zero. The stems which failed to recover were not included in the calculations. This treatment of the data is in general the same as that employed by Barth (1938a), and appears to give the most accurate index of regeneration rate.

Results

The results are summarized in Table I, and the rate of regeneration is plotted against pH at the different levels of oxygen concentration in

Table I

The interrelationship of oxygen and hydrogen ion concentration in influencing Tubularia regeneration

Initial pH	Change in pH	Initial O_2 cc./l.	Change in O_2 cc./l.	L micra	t hours	L/t
			Experiment 1			
8.00	−0.17	8.4	−1.0	1246	31.3	39.8
7.00	+0.03	8.8	−1.3	969	43.8	22.1
6.60	+0.11	8.5	−1.1	629	65.5	9.6
6.64	+0.08	7.9	−1.1	630	66.4	9.5
6.30	+0.13	8.2	−0.9	0	106.0	0
			Experiment 2			
8.03	−0.12	11.1	−2.2	1693	28.8	58.8
7.52	−0.10	10.3	−2.0	1491	34.4	43.3
7.33	−0.01	9.8	−1.4	1623	33.6	48.3
6.97	+0.15	10.5	−1.9	1571	37.5	41.9
6.74	+0.25	10.2	−1.7	1406	40.4	34.8
6.26	+0.04	9.7	−1.2	1085	68.0	16.0
			Experiment 3			
8.00	−0.08	10.3	−2.2	1346	30.6	44.0
6.96	+0.15	10.5	−2.4	1230	37.8	32.5
6.69	+0.17	9.9	−1.7	995	56.3	17.7
8.00	−0.08	5.6	−0.1	921	41.6	22.1
6.96	+0.13	5.3	+0.2	643	69.1	9.3
6.69	+0.16	5.1	+0.3	255	70.5	3.6
			Experiment 4			
8.07	−0.03	6.5	−0.7	1054	33.2	31.7
7.07	+0.28	6.3	−0.3	848	46.4	18.3
6.82	+0.28	6.0	−0.5	699	71.2	9.8
6.63	+0.25	6.0	−0.7	29	91.0	0.3
8.10	−0.04	3.4	+1.3	786	52.3	15.0
7.44	+0.14	3.5	+1.1	577	58.3	9.1
7.16	+0.26	3.5	+1.3	426	78.8	5.4
7.00	+0.21	3.6	+0.6	53	81.0	0.7
6.81	+0.17	3.4	+0.6	0	120.0	0
6.64	+0.15	4.0	+0.8	0	120.0	0

Figure 1. In Figure 1 and in the text the data are presented in terms of initial pH and initial oxygen concentration. The hydrogen ion concentrations remained fairly constant during the course of the experiments, the greatest pH change being 0.28 units. The greatest change

in oxygen occurred at the high oxygen concentrations. However, as shown by Barth (1938a), at high oxygen the rate of regeneration changes slowly as the oxygen is varied, so that this probably does not enter as an important factor. The pH range studied was from 8.10 (normal sea water) to pH 6.26. The levels of oxygen concentration used extended from approximately 3.5 to 10.5 cc. O_2 per liter (normal sea water contains 4.5–4.8 cc. O_2/l.).

As the pH of the sea water is lowered, the rate of regeneration falls off at all levels of oxygen concentration. At the highest level of oxygen concentration studied (Exp. 2; $O_2 = 10.5$ cc./l. approx.) there was a

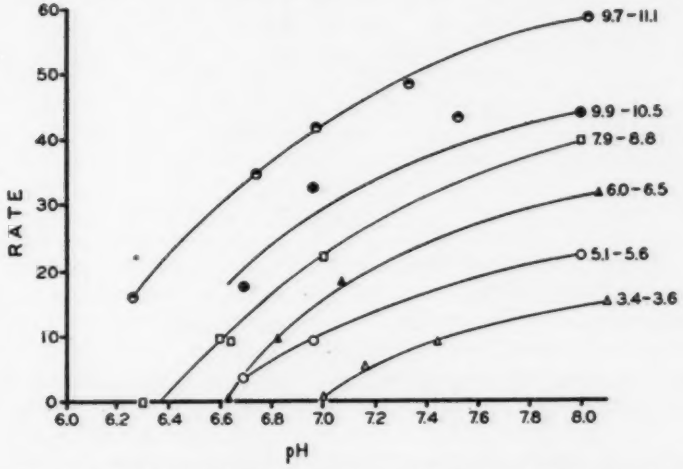

FIG. 1.[1] The interrelationship of oxygen and hydrogen ion concentration in influencing *Tubularia* regeneration. Data presented in Table I. Exp. 1 = □; Exp. 2 = ◓; Exp. 3 = ● and ○; Exp. 4 = ▲ and △. Rate = L/t where L = length of primordium in micra and t = time in hours. The pH values represent the readings at the beginning of the experiments. The figures to the right of each curve indicate the range of initial concentrations in cc./l.

fall of 42.8 R. U. as the pH was lowered from 8.03 to 6.26. At the lowest level of oxygen concentration (Exp. 4; $O_2 = 3.5$ cc./l. approx.), the fall was from 15 R. U. (pH 8.10) to 0 (pH 6.81). As the level of oxygen concentration is increased, however, the rate of regeneration is increased, not only at the pH of normal sea water but at all of the pH values studied. It should be noted that stems collected at different times, even though selected for uniformity, vary in their susceptibility

[1] The author wishes to thank Mr. Jack Godrich for his assistance in the preparation of the figure.

to oxygen changes (Barth, 1938a), so that the results of the individual experiments are only approximately comparable. The trend with increased oxygen is nevertheless quite clear. In the last two experiments the regeneration of stems from the same source was studied at two levels of oxygen tension so that the results are strictly comparable. Thus, in Experiment 3, increasing the oxygen concentration from 5 to 10 cc. O_2/l. increased the rate of regeneration 21.9 R. U. at pH 8.00, 23.2 R. U. at pH 6.96, and 14.1 R. U. at pH 6.69.

Complete inhibition of primordia formation may likewise be determined by both the oxygen and hydrogen ion concentrations of the sea water. Barth (1938a) found that the lower limit for regeneration in sea water at normal hydrogen ion concentration (pH 8–8.2 approx.) is between 0.35 and 1 cc. of oxygen per liter. As the hydrogen ion concentration is increased, complete inhibition occurs at higher oxygen concentrations (Table I; Figure 1). At pH 7.00 almost complete inhibition occurred at 1.6 cc. O_2 per liter, while at pH 6.30 complete inhibition occurred at 8.2 cc. O_2 per liter.

Discussion

An increase in the oxygen concentration of sea water results in an increased rate of regeneration. This observation was made by Barth (1938a), and is confirmed by the experiments reported here. However, as the hydrogen ion concentration is increased, there is a resultant decrease in the rate of regeneration. That the oxygen and hydrogen ion concentrations are interrelated in their effects is shown clearly by the results (Table I; Figure 1). The ability to form primordia is limited by the relative concentrations of both these factors in the sea water.

The inhibitory effect of low pH is not dependent upon the source of the hydrogen ion. In the experiments reported here the change in hydrogen ion concentration was brought about by increasing the carbon dioxide tension of the sea water. Inhibition is also obtained when the pH of the sea water is regulated by means of acid, base, and buffer (Goldin, 1942).

The interaction of these two environmental factors may likewise determine whether regeneration will occur or be completely inhibited. For, as the hydrogen ion concentration of the sea water is increased the oxygen concentration required for regeneration is also increased. Thus the complete inhibition of regeneration which has been obtained after various treatments is clarified. Morgan (1903) found that the cut end of a *Tubularia* stem fails to regenerate when it is placed in sand. Ligat-

ing the cut ends of stems inhibits regeneration. Barth (1938a) and Rose and Rose (1941) demonstrated that the covering of the cut end of a *Tubularia* stem with a glass capillary is sufficient to cause inhibition. Inhibition is also obtained when coenosarc fragments of *Tubularia,* as well as stem segments, are inserted in glass tubing (Goldin, 1942). The inhibition in all of these cases may be attributed to both lack of oxygen and increase of hydrogen ion concentration. The inhibition of stem regeneration in glass tubing occurred at pH 6.9, while stems kept in open dishes (4.4 cc. O_2/l.) failed to regenerate in the neighborhood of pH 6 (Goldin, 1942). It was suggested that this difference could be accounted for if, in the glass tubing, a decrease in the oxygen concentration of the sea water occurred concomitantly with the accumulation of acid metabolites. This interpretation is supported by the experiments reported here, for almost complete inhibition occurred at pH 7.0 when the oxygen concentration was reduced to 3.6 cc. O_2/l.

The results lend support to the conclusions of Goldin (1942) on the origin of polarity in *Tubularia* regeneration. The conditions of oxygen and hydrogen ion concentration which are required for regeneration may likewise account for the origin of polarity. The polarity of dedifferentiated coenosarc fragments (Goldin and Barth, 1941; Goldin, 1942) as well as stem segments (Komori, 1933; Miller, 1937, 1939) may be determined by subjecting them to a differential environmental exposure. The differential exposure may consist of a gradient of oxygen availability, a gradient of hydrogen ion concentration (or CO_2), or a combination of both of these factors. The effectiveness of the gradients would be dependent upon the resultant concentrations of both oxygen and hydrogen ion at the exposed surfaces of the coenosarc.

In order to proceed with studies of the chemical processes involved in regeneration it is necessary to understand fully the physiological action of these environmental factors. Oxygen apparently plays an important role in the respiratory mechanism involved in regeneration. Barth (1940) studied the oxygen consumption of *Tubularia* stems, and showed that there exists a close correlation between oxygen consumption and the rate of regeneration. As the oxygen tension is increased, the tissues consume more oxygen, and the rate of regeneration is increased. Further, distal levels of the stem regenerate more rapidly and have a higher rate of oxygen consumption than proximal levels. The close relationship between the effects of oxygen and hydrogen ion concentration indicates that the same processes are affected. In this connection it would be important to test whether increasing the hydrogen ion concentration (or carbon dioxide tension) of the sea water decreases the rate of oxygen consumption.

SUMMARY

The regeneration of *Tubularia* stem segments was studied in sea water at varying concentrations of hydrogen ion (carbon dioxide tension) and oxygen. As the hydrogen ion concentration was increased there was a fall in the rate of regeneration. On the other hand, as the dissolved oxygen was increased, the rate of regeneration was increased.

The effects of hydrogen ion and oxygen are interrelated, the resultant rate of regeneration being determined by the relative concentrations of both these factors in the sea water.

Complete inhibition may be effected by increased hydrogen ion, decreased oxygen, or by a combination of both of these factors. As the hydrogen ion concentration was increased, complete inhibition occurred at higher oxygen concentrations.

The origin of polarity in *Tubularia* regeneration was discussed in terms of these results.

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THE EFFECTS OF TEMPERATURE GRADIENTS ON THE PUPAL–ADULT TRANSFORMATION OF SILKWORMS

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There is good evidence that the adult development of the higher holometabolous insects is evoked by one or more factors arising in the anterior end of the larva and pupa (Hadorn, 1941; Scharrer, 1941). In the present investigation further information was sought concerning the possible action of such a differentiation center in the pupal-adult transformation of silkworms (Williams, 1940). By the application of temperature gradients, one end of the pupa could be exposed to a temperature ordinarily high enough to induce development and the other end to a temperature ordinarily low enough to maintain dormancy. The developmental dependency of the two ends and of the various tissues could thus be tested.

There is apparently only one previous record of the use of temperature gradients in studying insect development; namely, the investigation of Giersberg (1929) concerning the pigmentation of Lepidoptera.

Materials and Methods

The experiments were performed on two species of native silkworms, *Samia walkeri* (*cynthia*) (F. & F.) and *Telea polyphemus* (Cram.). The diapausing pupae were collected during winter, after having been exposed to low temperature, and were stored at 3° to 5° C. until used. Since escape from diapause is facilitated by prolonged exposure to cold (Uvarov, 1931), it may be presumed that the pupae were in a physiological state to develop when heated. A further advantage to the use of such material was the fact that all individuals at the beginning of the experiments were at the uniformly low degree of adult differentiation characteristic of diapausing pupae.

The apparatus consisted of an incubator from which the glass in the hinged door had been removed and substituted by a corrugated cardboard diaphragm. The pupae, in various orientations, were fitted snugly into holes of suitable size in this partition, the incubator set at a favorable developmental temperature (25° to 30° C.), and the entire

apparatus placed in a cold-room having a temperature of 3° to 5° C. A beaker of water inside the incubator maintained the humidity at a high level.

Most of the animals were oriented with the diaphragm midway of their longitudinal axes. There was thus established a temperature gradient along the body of each individual. Fortunately, no major neutralization of this effect would be expected from the circulation of the blood due to the very rudimentary state of this system in the diapausing pupa.

The animals were exposed to the temperature gradient for a maximum of 139 days. During this time the pupae which showed signs of development were removed and studied. Most of the animals were photographed and fixed in Bouin's solution. A few were exposed to room temperature to test its effect on subsequent development.

Experimental Results

Head and Thorax Heated—Abdomen Chilled

As can be seen from Table I, development took place in all pupae thus oriented, with the exception of two individuals that died. Striking differences, however, were found in the degree of adult differentiation at the two ends. After exposure to the temperature gradient for

Table I

Development attained by pupae exposed to longitudinal temperature gradients

	Front heated; abdomen chilled	Abdomen heated; front chilled
No development	0	6
Front moulted; abdomen immature	21	0
Abdomen moulted; front immature	0	5[1]
Both ends immature		
Front more mature than abdomen	4	0
Abdomen more mature than front	0	15
Dead	2	2
	27	28

[1] Only the anterior ¼ to ⅓ of pupae chilled.

an average of 58 days most of the animals moulted the pupal cuticle at the anterior end posteriorly to about the second abdominal segment. The rest of the abdomen remained firmly enclosed within the pupal cuticle from which it could be removed only by tearing the body wall. The anterior ends of these animals were wholly adult, except that the

wings were not expanded. All the adult structures of the head and thorax were well differentiated and pigmented, as shown in Figure 4. These pupal-adult animals could walk in normal fashion and move the wings slowly. Four individuals were removed from the diaphragm before the anterior end had completely transformed.

Dissection of all of the animals that had been oriented in this manner showed that some adult development had also occurred in the chilled abdomen. However, the degree of adult differentiation of the abdomen was in all cases less than that of the anterior region. The large pupal midgut was frequently still present, although modifications of the gut in the adult direction had clearly taken place. For example, slight convolutions had appeared in the straight hindgut of the pupa and a terminal rectal expansion had been differentiated. The genitalia had initiated development, but were still immature. The first stages in the formation of the adult abdominal cuticle were apparent, although the abdominal muscles were still attached to the pupal cuticle.

Thus it is clear that development in the adult direction had begun at both ends in animals heated anteriorly and cooled posteriorly. The anterior warm end attained full or extensive development whereas the chilled posterior end remained in an earlier stage of adult differentiation.

Head and Thorax Chilled—Abdomen Heated

Six of the 28 pupae oriented in this fashion underwent no development whatsoever, although their abdomens were exposed to the warm temperature for an average of 97 days. At the end of this time these individuals were still alive, but unchanged externally and internally (Fig. 1).

Twenty pupae initiated development. For most of these animals adult differentiation was still incomplete at both ends after an exposure to the temperature gradient for an average of 74 days. Here again the warm end showed the more extensive adult differentiation, although the contrast between the developmental conditions at the warm and cold ends was not as great as when the gradient was the reverse. The adult cuticle of the abdomen was in most cases well chitinized, while that of the head and thorax less mature. Furthermore, fully developed adult genitalia occurred on animals whose thoracic muscles were incompletely formed. It may be noted also that in every case where the earliest stages of differentiation of the adult had begun in the abdomen, adult development had also been initiated in the head and thorax.

In only five instances did the abdomen become wholly mature and moult during the term of the experiments. These animals had been

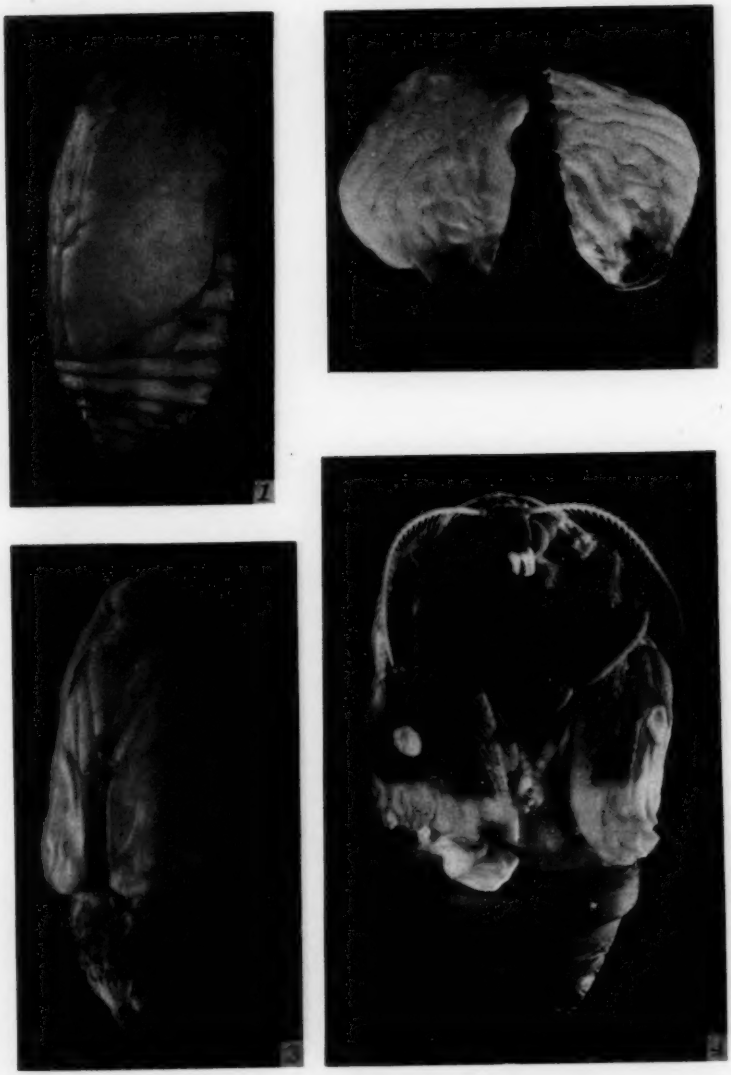

PLATE I

treated with a less severe temperature gradient, however, since only one-fourth to one-third of each individual was placed on the cold side of the diaphragm, in contrast to the usual orientation of the diaphragm midway of the longitudinal axis. The pupal cuticle of the abdomen became thin and transparent in these five animals so that it was easily removed, whereas the cuticle of the head and thorax remained firm and could be dissected off only with difficulty. A comparison of the opposite ends showed that the anterior region had attained the adult condition also, except that pigmentation and finer histological maturation were generally absent. As shown in Figure 3, all the adult structures of the head and thorax were present but generally soft and incompletely chitinized. The heated abdomen had completely transformed into the adult condition.

In summary, the results obtained from animals chilled anteriorly and heated posteriorly indicate that the development of the abdomen is retarded or prevented by cooling the head and thorax. In those cases where the abdomen became fully developed and moulted, the head and thorax were in the last stages of adult differentiation.

One Side Chilled—Other Side Heated

Pupae treated in this manner produced animals which were wholly adult, except that development proceeded somewhat more rapidly on the warm side. This was especially clear in regard to the degree of pigmentation: the hot side attained complete pigmentation before this process had scarcely begun on the chilled side. A conclusive test of this orientation was impossible, however, due to the difficulty in establishing a sufficiently steep temperature gradient transversely through the relatively narrow pupa.

PLATE I

FIG. 1. Diapausing pupa of *S. walkeri*.

FIG. 2. Hind wings of an individual (*S. walkeri*) that had been heated posteriorly and chilled anteriorly. The wing tips, which were on the warm side of the diaphragm, have become pigmented, whereas the chilled anterior region remains in an earlier developmental condition.

FIG. 3. Result of chilling the anterior region of *S. walkeri* and heating the posterior region. Both ends have transformed, but the chilled anterior region is less mature than the heated abdomen.

FIG. 4. Result of heating the anterior half of *T. polyphemus* and chilling the posterior half. The heated region has completely transformed and moulted, whereas the chilled end remains firmly attached to the pupal cuticle. The wings and legs also show gradients in development.

Subsequent Effects of Developmental Temperature

Certain of the animals that had moulted the pupal cuticle at one end were removed from the diaphragm and placed at room temperature. In about ten days a second partial moult occurred in which the rest of the pupal cuticle was shed to produce the complete adult.

At such a time those animals which had previously moulted the abdominal cuticle showed curious movements of the abdomen: waves of contraction passed forward along its length in a manner similar to that which occurs during the normal eclosion of the adult and expansion of the wings. The wings in no case were expanded, however.

Thus it was possible to produce the complete adult by two partial moults.

Local Action of Temperature

In most of the pupae the diaphragm passed across the wing flaps with the result that the tip of each wing was exposed to one temperature and the base to the other extreme. Development proceeded normally on the heated end, whereas the chilled end remained in an earlier stage of development. This was especially clear in regard to pigmentation, as shown in Figures 2 and 4. The pigmentation of the heated portion was generally complete and normal in every respect up to a narrow transitional zone of incomplete pigmentation at the former level of the diaphragm.

Other examples of local temperature action were found along the lengths of the legs and the antennae.

Discussion

An analysis of the effects of temperature gradients gives us some information in regard to the initiation of adult formation after diapause. We have noted that all of the animals which began to transform showed more rapid differentiation at the warm than at the cool end; yet in no instance did development occur in the heated region without some indication of development likewise being present in the chilled region. This observation, that dormancy is apparently terminated simultaneously over the entire animal, suggests the existence of some mechanism which evokes the formation of the adult.

The experimental results permit a rough localization of such a stimulating factor. Without exception, development took place in the surviving pupae whose heads and thoraces were heated. From this we may infer that dormancy is not maintained by cooling the abdomen. In

contrast, when the anterior end was chilled, 21 per cent of the individuals underwent no development whatsoever after 97 days: overall dormancy thus persisted in these animals when the head and thorax were inhibited by the low temperature. These results may be most simply explained in terms of the existence in the anterior end of the pupa of a developmental center which must become active for adult transformation to ensue.

Further evidence in favor of the presence of such a center is given by the excessive time necessary for the development of the remaining 79 per cent of the animals chilled anteriorly. These individuals usually showed only slight differentiation after an average of 74 days. On the contrary, the head and thorax when heated became fully transformed in the majority of animals after 58 days. The fact that 21 per cent of the specimens chilled anteriorly failed to begin development after prolonged exposure suggests that the longer time necessary for adult differentiation of all animals thus treated was primarily due to a delay in the initiation of development rather than to a retardation once it had begun. If the developmental center is in the anterior end of the pupa, such a delay would be expected.

This evidence in support of the existence of an adult-differentiation center is in agreement with the results obtained by a number of previous investigators using other techniques, as recently reviewed by Bodenstein (1939). It may be noted that the action of a differentiation center in the lepidopterous pupa has been found for both diapausing (Bytinski-Salz, 1933) and non-diapausing (Hachlow, 1931; Piepho, 1938a and b; Bodenstein, 1938, 1939) types of development. The results of the present investigation indicate that the differentiation center does not exert its effect in diapausing silkworms until after a prolonged period during which development is suspended. Such an extensive delay is not found for non-diapausing species and individuals. In view of this fundamental difference it is possible that diapausing and non-diapausing developments differ primarily in terms of the time at which the center attains an activity sufficient to evoke adult development.

The local effects of temperature gradients are also of interest in regard to certain of the differentiation processes which are generally considered to be under hormonal "control." For instance, there is considerable evidence from previous studies that moulting results from the action of a hormone arising in the head of the animal (Wigglesworth, 1939; Scharrer, 1941). It is therefore noteworthy that the majority of the animals heated anteriorly moulted the pupal cuticle of the head and thorax without shedding that of the posterior abdominal region.

This local action of hormonal factors is further illustrated by those wings which showed a gradient in pigmentation. The pupal wing-flaps correspond to flattened sacs into which the blood enters from the thorax by anterior openings at their bases. Hence the chemical environment of the wing tissues, in terms of the blood, must be uniform. Now it is well known that the pigmentation of the wings involves the reaction between the scales and a chromogen circulating in the blood (Braun, 1939). Nevertheless, under the influence of temperature gradients the pigmentation generally became complete in the heated region before the process had scarcely begun in the chilled region (Figs. 2 and 4). This phenomenon was also observed by Giersberg (1929) after various regions of the wing-flaps were chilled by cold water passed through tubing.

This evidence in regard to the local action of the chemical environment shows that, at least in these cases, hormones are the servants rather than the masters of development: the ultimate result is determined by the ability of the tissues to react.

Summary

1. Diapausing pupae of the silkworms, *Samia walkeri* (*cynthia*) (F. & F.) and *Telea polyphemus* (Cram.), were placed in a diaphragm and one end exposed to a temperature of 25° to 30° C. and the other end to a temperature of 3° to 5° C. There was thus established a longitudinal temperature gradient in each individual.

2. Development took place in the majority of animals thus treated. For each of these pupae evidence was found that the termination of diapause occurred simultaneously throughout the animal. This observation suggests the existence of a developmental center which evokes the overall initiation of adult differentiation.

3. An analysis of the results obtained when the head and thorax were heated with those obtained when this region was chilled permits a rough localization of such a developmental center in the anterior end of the pupa. Since a similar relationship has been noted for non-diapausing pupae by previous investigators, it is possible that diapausing and non-diapausing developments differ primarily in regard to the time at which the developmental center attains an activity sufficient to stimulate adult differentiation.

4. After the pupal-adult transformation has once been initiated, the tissues are capable of a high degree of developmental autonomy. Animals were produced which were wholly mature at the heated end while the cold end remained in an earlier stage of adult formation. This result was also observed within single organs. In such cases the develop-

mental velocity of each region was related to the local metabolic conditions as affected by the temperature gradients.

5. Of particular interest were the effects of temperature gradients upon moulting and pigmentation, processes that are generally recognized as involving the action of chemicals of a hormonal nature. The contrasting results observed in the hot and cold regions, notwithstanding their uniform blood supply, emphasizes the importance of tissue competency in carrying out hormonal reactions.

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RESPIRATION OF A COLORLESS FLAGELLATE, ASTASIA KLEBSII

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Introduction

There is a growing literature on protozoan respiration recently reviewed by Jahn (1941), but the sum total of research done in this field is still quite small and not comparable to the work done on respiration of bacteria, yeast and metazoan tissues. In the belief that a detailed study of the respiratory characteristics of a flagellate would have some value from the standpoint of comparative physiology, the present work was undertaken. This paper deals with the effects of various factors on the respiration of *Astasia klebsii.*

Acknowledgments

I am indebted to Professor K. C. Fisher, who guided and helped me in every possible way; to Professor W. J. Kostir for suggestions and criticisms; to Professor B. S. Meyer for the use of his Warburg apparatus in a number of the experiments; and to Professor D. R. Goddard for a supply of hexosediphosphate.

Materials and Methods

Astasia klebsii, the species used in this investigation, is a free-living, colorless, plantlike flagellate. Its nutrition is saprozoic. Food reserves of paramylum, a starch-like polysaccharide which does not respond to the usual iodine test for starch, are stored as small granules in the cell. The same strain employed in a previous investigation (Von Dach, 1940) was used.

Pure (bacteria-free) clone cultures of *Astasia klebsii* were grown at 27° C. in one liter Erlenmeyer flasks containing 500 cc. of the following organic medium:

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[2] Muellhaupt Scholar, 1939–1940.

KNO_3	0.5 gm.
KH_2PO_4	1.5 gm.
$MgSO_4 \cdot 7H_2O$	0.1 gm.
NaCl	0.1 gm.
$CaCl_2$	0.01 gm.
$FeCl_3$	trace
Sodium acetate	2.0 gm.
Bacto-tryptone	5.0 gm.
Distilled water	1000 cc.

(Adjusted to pH 5.8 with N/1 NaOH or N/1 HCl)

After incubation the cells were concentrated by centrifuging, washed three times in inorganic buffer by centrifuging, and suspended in buffer. The buffers used were potassium phosphate mixtures (M/20 K_2HPO_4–KH_2PO_4) containing the following added ingredients per liter: KNO_3 0.09 gm., $MgSO_4 \cdot 7H_2O$ 0.09 gm., NaCl 0.09 gm., $CaCl_2$ trace, $FeCl_3 \cdot 6H_2O$ trace. The proportions of K_2HPO_4 and KH_2PO_4 were varied according to the pH desired, after Sorenson's tables (Peters and Van Slyke, 1932, p. 816). The pH of the buffers was checked by a Hellige colorimetric comparator or a glass electrode. It was found that the cells survived longer and with less deterioration in these buffers than they did in M/20 phosphate alone.

Respiration of the cell suspensions was measured by the Warburg direct method. The standard procedures of this method, as described by Dixon (1934), were employed.

All the experiments were conducted at 25.2° C. ($\pm$ 0.05°). The vessels were shaken continuously at from 70 to 90 cycles per minute, through an arc of from 4.5 to 7.5 cm.; these rates were found to be wholly adequate.

A portion of each cell suspension was fixed in 10 per cent formalin for determining cell concentration. Cell counts were made by means of a Sedgwick-Rafter counting cell and a Whipple ocular micrometer. Usually between four and eight million cells per vessel were used.

At the end of an experiment the cell suspensions were removed from the respirometer vessels for the following tests: (1) pH determination; (2) microscopic examination for possible cell injury and bacterial contamination; (3) in some cases, cell counts.

In testing for cell injury and death a modification of the method of Devereux and Tanner (1927) was used. Two or three drops of cell suspension were mixed with a drop of 0.5 per cent erythrosin on a slide and examined. Cells which did not stain and which displayed movement, whether swimming or protoplasmic contraction, were regarded as uninjured. Cells which did not move at all and which nearly always

became stained were regarded as irreversibly injured or dead. Repeated tests have shown the validity of this criterion for *Astasia*.

No increase in cell number was observed to occur during an experiment. Except where otherwise noted, the number of injured astasias remained negligible, and the pH of the suspensions remained constant within 0.2 or 0.3 unit, throughout the experiments.

The number of bacteria present in the cell suspensions was negligible in all cases. While slight bacterial contamination undoubtedly occurred as soon as the pure cultures were exposed to the air, the process of quickly washing the cells and suspending them in inorganic buffer kept bacterial growth at a minimum. These experiments, then, may be regarded as essentially bacteria-free.

The gas space of the vessels contained air in all cases. Most of the experiments were conducted at pH 5.8; this pH had previously been found most favorable for growth (Von Dach, 1940). Most of the experiments were of two or three hours' duration. Results were calculated as cubic millimeters of oxygen consumed per hour per million cells, or as cubic millimeters of carbon dioxide produced per hour per million cells. In nearly all cases, the values for duplicate respirometers did not differ from each other by more than 7 per cent.

Results

I. *Respiration in Inorganic Medium*

The growth of *Astasia klebsii* cultures was found to be similar to that of yeast and bacteria. In newly-inoculated cultures there occurred in succession: (1) the "logarithmic growth phase" (which usually lasted about 120 hours), during which growth proceeded at a constant maximal rate until a cell concentration of about 300,000 cells per cc. was reached; (2) the "phase of negative growth acceleration," during which growth continued briefly at a decreasing rate; (3) the "stationary phase," during which the cell population remained fairly constant at approximately 650,000 cells per cc. for at least five weeks, with no appreciable amount of cell division or cell death.

In these experiments it was not convenient to regulate the initial cell concentrations of the culture; since duration of the logarithmic phase must increase as the initial cell concentration is decreased, it would not be enough to merely state the ages of the various cultures here. Classification of cultures as to phase of growth was based on a study of their growth curves.

Figure 1 shows the relation of rate of oxygen consumption in inorganic medium to time. Experiments on several cultures in the loga-

rithmic phase and several in the stationary phase are represented. These data show the following general relationships:

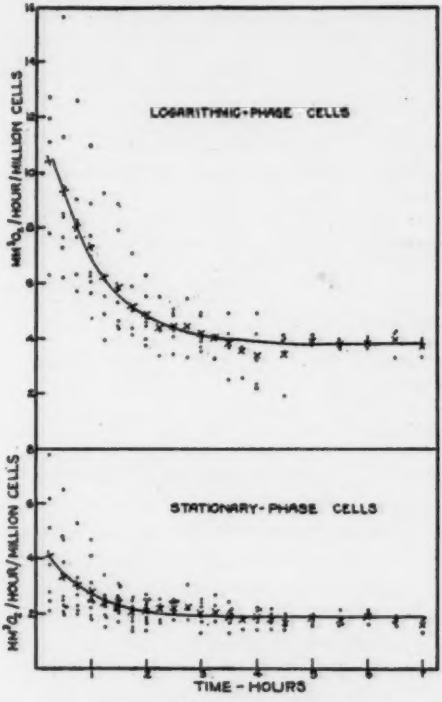

FIG. 1. Change in rate of respiration in inorganic buffer with time (pH 5.8). Dots, values from separate experiments; crosses, average values. (Logarithmic-phase cells from cultures 86 to 162 hours old; stationary-phase cells from cultures 188 to 504 hours old.)

For both old and young cells, an early period of declining respiration was followed by a more or less constant period. In logarithmic-phase cultures (i.e. "young" cells) there was an average oxygen consumption of 10 cu. mm. per hour per million cells for the first observation (at 15 minutes). The rate of respiration declined sharply for two hours, then remained fairly constant at about 3.8 cu. mm. O_2 per hour for several hours. In stationary-phase cultures (i.e. "old" cells), on the other hand, the initial average rate of oxygen consumption was 4 cu. mm. per hour per million cells; the rate declined gradually for two hours, then re-

mained fairly constant at a value of 1.9 cu. mm. O_2 per hour for several hours. Respiration rates differed somewhat even in cultures of the same age and growth phase.

Oxygen consumption in inorganic buffer was found to be approximately the same at hydrogen-ion concentrations between pH 4.5 and pH 7.9. (Results of typical experiments are shown in Table 1.)

TABLE 1

Effect of pH on respiration

(Duration of experiments three hours; M/40 sodium acetate used)

Exp. no.	Growth phase of cells	Oxygen consumption as cu. mm. O_2/hour/million cells					
		pH 4.5 inorganic acetate		pH 5.8 inorganic acetate		pH 7.9 inorganic acetate	
1	Logarithmic	4.59		4.91		5.20	
2	Stationary	2.49		2.43		2.48	
3	Stationary		5.75*	2.63	31.39		
4	Stationary			2.24	28.09		29.28

* Much cell injury.

Several experiments were performed to determine the respiratory quotient in inorganic buffer at pH 5.8. Most of the determinations of rate of carbon dioxide production were carried out without the customary addition of acid (see Dixon, 1934), since it was found that there was no appreciable accumulation of retained carbon dioxide in the cell suspensions under the conditions of these experiments. The respiratory quotient was usually found to be slightly less than one. (R. Q. values obtained in four three-hour experiments: 0.86, 0.92, 0.95, 1.04.)

II. *Effects of Various Organic Compounds*

In order to throw light on the nature of the substances and reactions involved in the respiratory metabolism of *Astasia*, the effect of various organic compounds on oxygen consumption was studied. Most of the substances tested serve as substrates for many types of cells; others are known to play some accessory part in certain cellular respiration systems. (See Needham and Green, 1938, for a recent account of the role of these compounds in cellular respiration.)

Usually 0.18 cc. of a M/2 test solution was added to 1.62 cc. of cell suspension in a Warburg vessel, and the respiration compared to that of control vessels containing cells suspended in inorganic buffer alone. Cultures of various ages were employed in these experiments. In most cases cultures in the stationary phase of growth were used. In each

separate experiment, oxygen consumption in the presence of the tested compound was expressed in terms of percentage of the control respiration. Results are shown in Table 2.

A definite increase in oxygen consumption was produced by the following: sodium formate, sodium acetate, sodium propionate, and ethyl alcohol (all M/20 concentrations), and M/40 sodium hexosediphosphate. Acetate had the most marked effect, with propionate and ethyl alcohol producing somewhat smaller increases. Rather slight but consistent in-

TABLE 2

Effect of various organic compounds on respiration

(M/20 concentrations used unless stated otherwise. Nearly all experiments of two hours' duration.)

Substances tested	Respiration as per cent of that in inorganic medium in separate experiments	Respiration as per cent of that in inorganic medium (average)
Sodium formate	192; 147; 151; 137	157
Sodium acetate	488; 731; 1130	783
Sodium propionate	428; 328; 229	328
Sodium butyrate	492;* 174;* 46;* 66*	195*
Sodium hexosediphosphate (M/40)	172; 163	168
Ethyl alcohol	833; 355; 278	489
Sodium citrate	115; 68; 99	94
Sodium beta-glycerophosphate	120; 86; 96	101
Sodium lactate	111; 113; 106	110
Sodium succinate	118; 96; 103	106
Galactose	132; 100; 110	114
Levulose	102; 113; 84	100
Glycerol	110; 127; 105	114
Dextrose	98; 107; 104	103
l-Xylose	96; 103; 129	109
Methyl alcohol	99; 127	113
Ethylene glycol	102	102
Potassium malonate (M/500)	100	100
Potassium fumarate (M/500)	99	99

* Much cell injury.

creases in respiration occurred in the presence of formate and hexosediphosphate. Inconsistent results were obtained with M/20 sodium butyrate; in some experiments it produced an increase in respiration, in other cases a decrease. Butyrate always caused injury or death in from 50 per cent to 90 per cent of the total cells present; the greater degree of injury was correlated with lower oxygen consumption. Various common substrates which are oxidized by many types of cells, such as simple sugars, lactate, succinate, etc., had no appreciable effect on the respiration of *Astasia*.

Since acetate produced the greatest increase in oxygen uptake, and since it has been shown to greatly increase growth of plantlike flagellates in general (Hall, 1941) and of *Astasia* in particular (Von Dach, 1940), the effects of this substance on respiration were studied in some detail.

Respiration in Acetate

At pH 5.8, when a solution of sodium acetate was tipped from the side-arm of a Warburg vessel into the main compartment containing

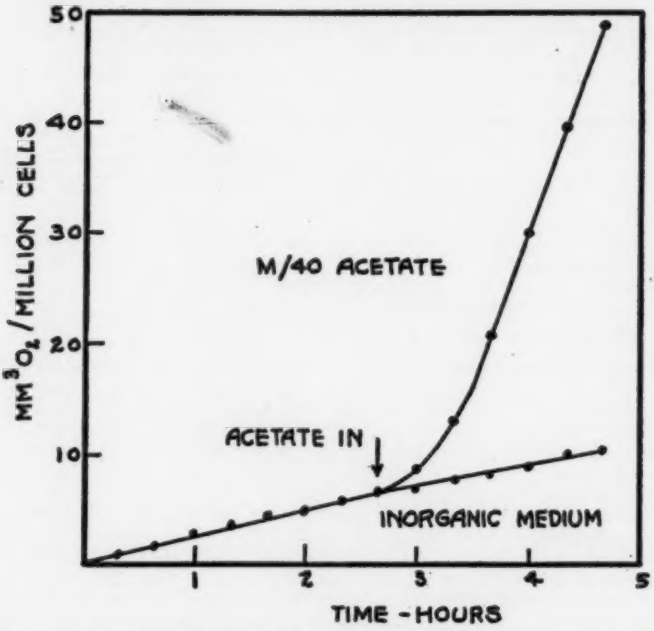

FIG. 2. Effect of adding acetate to cells in inorganic medium at pH 5.8. Results of a typical experiment. First 160 minutes: curve represents average respiration in four vessels containing cells in inorganic medium. M/40 acetate was added to two of the vessels at 160 minutes, at the arrow mark. From 160 to 280 minutes: upper curve represents average respiration in the two vessels to which acetate was added, lower curve represents average respiration in the two control vessels to which nothing was added.

cells in inorganic buffer, the rate of respiration rose steadily during a period of about one hour. (See Figure 2.) Thereafter respiration usually continued at a constant maximal rate for several hours. The acetate respiration data in this section deals with constant maximal rates.

Oxygen consumption in acetate was approximately the same at pH 5.8 and pH 7.9; but at pH 4.5 respiration was much less, and there was marked cell injury (Table 1). This differs from respiration in buffer alone, where oxygen consumption was approximately the same over the range pH 4.5–pH 7.9.

At pH 5.8 oxygen consumption was for a time approximately the same at acetate concentrations between M/20 and M/80 (see typical results in Table 3). However, after some time respiration in M/80 acetate declined rapidly, while that in M/20 and M/40 remained constant. It may be presumed that the enzyme system concerned was at first "saturated" with substrate at all these concentrations but as acetate was used up, the concentration in the M/80 vessels fell below the critical concentration for enzyme saturation.

TABLE 3

Effect of acetate concentration on oxygen consumption of logarithmic-phase cells
(Initial pH 5.8)

Time period	Control (inorganic buffer)	M/10 acetate	M/20 acetate	M/40 acetate	M/80 acetate
	cu. mm. O_2/hour/million cells				
0 to 60 min.	5.72	39.28	55.50	62.36	64.27
60 to 120 min.	4.42	44.46	61.40	67.52	69.12
120 to 180 min.	4.01	46.96	64.88	68.39	19.23
180 to 240 min.	3.37	48.62	66.58	69.56	4.80

Respiration in M/10 acetate was always markedly lower than in more dilute acetate concentrations (Table 3), although cell injury occurred in only one out of five experiments.

The growth phase of cells had a definite effect on their respiration in acetate. In numerous experiments it was found that in acetate medium, logarithmic-phase cells consumed from 40 to 60 cu. mm. O_2 per hour per million cells; while stationary-phase cells usually consumed from 20 to 30 cu. mm. O_2 per hour per million cells.

Experiments were performed to determine the respiratory quotient in acetate. In the CO_2 determinations bound carbon dioxide was liberated from the cell suspensions by tipping in 5N H_2SO_4 from the sidearms of the vessels (Dixon, 1934). The respiratory quotient in acetate was found to be approximately 1. (R. Q. values obtained in two experiments: 1.04, 1.03.) This value corresponds to the reaction for the complete oxidation of acetic acid to carbon dioxide and water:

$$CH_3COOH + 2O_2 \rightarrow 2CO_2 + 2H_2O.$$

The pH of cell suspensions in acetate usually increased somewhat (0.5 to 1.0 pH unit) during the course of an experiment.

No chemical tests were made to determine whether acetate was actually used up in the cell suspensions during these experiments. However, chemical analyses of old culture media have shown that the acetate concentration in cultures decreases with time (unpublished data). Hence it is reasonable to presume that the increase in respiration produced by acetate is due to the utilization and oxidation of this substance by the cells, rather than to some catalytic stimulating effect.

III. *Tests for Cytochrome*

The distribution of cytochrome among the Protozoa has received little attention; only a few records of its occurrence in members of this phylum have been published (cited by Jahn, 1941; Baker and Baumberger, 1941).

To determine whether cytochrome was present in *Astasia klebsii,* the usual method of spectroscopic examination was used. A very concentrated suspension of cells in acetate medium was placed in a small glass vial and examined by means of a Leitz micro-spectroscope. Three absorption bands were seen, located at approximately 605 mμ, 565 mμ, and 555 mμ; these values agree fairly well with the generally reported locations of bands for cytochromes a, b, and c respectively (see Oppenheimer and Stern, 1939). Further evidence that these absorption bands were caused by cytochromes was furnished by the following observations:

(1) The bands disappeared after vigorous aeration of the suspension, then gradually reappeared and regained full intensity. (Aeration converts reduced cytochromes to oxidized cytochromes, which return to the reduced form when reducing conditions again prevail.)

(2) Addition of M/100 NaCN to the cell suspension did not change the absorption spectrum, but did prevent removal of the absorption bands by aeration. (Cyanide inhibits cytochrome oxidase, so that the cytochromes perforce remain in the reduced form.)

The oxidation of para-phenylene-diamine (abbreviated as PPD) has often been used as a test for the cytochrome-cytochrome oxidase complex, although this test is not wholly specific nor unambiguous (see Stotz, 1939, and Hogness, 1939). The effect of various concentrations of PPD upon oxygen consumption of *Astasia* was tested in several experiments. In a single instance M/200 PPD produced a 70 per cent increase in respiration and did not injure the cells; in all other experi-

ments, at PPD concentrations between M/20 and M/1000, the respiration was equal to or below the control respiration and nearly always extensive cell injury occurred.

IV. *Effects of Respiratory Inhibitors*

A. Cyanide

Cyanide is generally regarded as a useful tool for determining how much of the respiration of any organism is carried on through the cytochrome-cytochrome oxidase system, and has so been used in studies on numerous types of cells and tissues (review by Commoner, 1940). The spectroscopic detection of cytochrome in *Astasia klebsii* made it seem

TABLE 4

Effect of M/100 NaCN on the increase in respiration produced by various substrates at pH 5.8

Substrate tested	Exp. 1 Ethyl alcohol (M/20)	Exp. 2 Sodium formate (M/20)	Exp. 3 Sodium acetate (M/40)	Exp. 4 Sodium propionate (M/20)	Exp. 5 Sodium hexosediphosphate (M/40)
	cu. mm. O_2/hour/million cells				
Respiration in inorganic buffer before adding substrate (control)	2.93	5.73	5.40	5.73	5.71
Respiration after adding substrate	8.15	8.45	41.43	18.81	8.41
(% of control)	(278%)	(147%)	(767%)	(328%)	(163%)
Respiration after adding M/100 cyanide to cell suspension in substrate	3.31	3.35	1.93	3.60	5.37
(% of control)	(113%)	(59%)	(36%)	(63%)	(94%)

likely that the cytochrome-cytochrome oxidase system played an important part in its respiration. Experiments on the effect of cyanide were accordingly undertaken.

Preliminary experiments were performed to determine the effect of cyanide on respiration in solutions of those substrates which had previously been found to increase oxygen consumption. It was found that the increase in respiration rate produced by formate, acetate, propionate, hexosediphosphate, and ethyl alcohol was usually completely abolished by M/100 sodium cyanide (Table 4).

Subsequently more detailed experiments on cyanide effect on respiration in inorganic buffer and in acetate medium were performed. The

cyanide solutions used were prepared by diluting M/1 NaCN with inorganic buffer of the same pH as the cell suspensions. The cyanide-KOH mixtures described by Krebs (1935) were employed as carbon dioxide-absorbing fluids, to prevent the escape of HCN from the cell suspensions during the course of an experiment.

Cyanide inhibition of respiration, both in inorganic buffer and in acetate, was found to be approximately the same at pH 5.8 and pH 7.9.

Cyanide inhibition of respiration is essentially a reversible reaction. It was necessary to show that cyanide did not visibly damage the cells, and that respiration went back to normal after removing the cyanide, before concluding that the only effect of cyanide was inactivation of the cytochrome-cytochrome oxidase system. Experiments along these lines were performed, employing cyanide solutions of various concentrations.

TABLE 5

Recovery of respiration from cyanide inhibition
(Logarithmic-phase cells in inorganic buffer, pH 5.8)

NaCN concentration	Respiration in cyanide (2 hours)	Recovery respiration (after removal of cyanide by washing) (2 hours)
	cu. mm. O_2/hour/million cells	
0	7.76	3.52
M/250	5.11	7.08
M/100	3.40	7.61
M/50	2.39*	5.20*

* Slight cell injury.

Using the erythrosin test, it was found that M/20 NaCN injured all astasias present within two hours. M/50 concentration usually injured a small percentage of the cells present. In M/100 cyanide there was scarcely any cell injury after four hours.

"Recovery" experiments were performed as follows: After a two-hour run in cyanide, the cell suspension in each Warburg vessel was removed and separately washed four times by centrifuging in inorganic buffer to remove the cyanide, then returned to the washed Warburg vessel for further observations on respiration. Results of a typical recovery experiment are shown in Table 5. These results show that when cells which had been in M/100 and M/250 cyanide were washed cyanide-free their rate of respiration rose to approximately the initial control level; for cells which had been in M/50 cyanide the recovery rate was somewhat below the initial control. Since M/50 cyanide proved somewhat toxic, it was not used in later experiments.

Figure 3 shows the results of a number of experiments on the effect of various concentrations of cyanide on respiration in inorganic buffer and in acetate, for logarithmic-phase cells and for stationary-phase cells. The experiments were performed at pH 5.8, using cyanide concentrations between M/100 and M/500,000.

For stationary-phase astasias under maximum cyanide inhibition (in M/100 cyanide), it was found that the cyanide-stable respiration was practically the same whether substrate was present or absent. In these

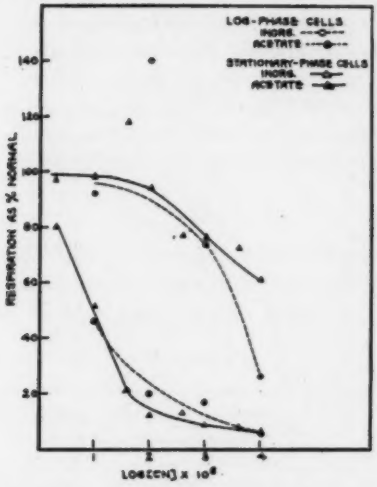

FIG. 3. Effect of different cyanide concentrations on respiration. Average values from three logarithmic-phase experiments and four stationary-phase experiments are represented (two-hour experiments at pH 5.8).

Normal respiration (average) as cu. mm. O_2/hour/million cells:

Log-phase cells in inorganic medium, 6.30
Log-phase cells in M/40 acetate, 46.96
Stationary-phase cells in inorganic medium, 2.28
Stationary-phase cells in M/40 acetate, 24.63

experiments, the average cyanide-stable respiration (expressed as cu. mm. O_2/hour/million cells) was 1.37 in inorganic buffer and 1.46 in acetate. In inorganic medium the total (cyanide-free) respiration was small, so that the percentage of inhibition by M/100 cyanide was relatively slight. But when substrate in sufficient concentration (M/40 sodium acetate) was present the cyanide-sensitive system presumably functioned at full capacity, a high total (cyanide-free) respiration was attained, of which the cyanide-stable portion formed only a small frac-

tion—consequently over 90 per cent of the respiration of substrate-saturated cells was cyanide-sensitive. For logarithmic-phase cells in M/100 cyanide the situation was much the same. The foregoing is in agreement with Commoner's (1940) statement: "Since most variations in the total rate of respiration are mainly due to variations in the activity of the cyanide-sensitive system alone, percentage inhibition by cyanide (i.e. "cyanide-sensitivity") increases with the normal rate of respiration."

Cyanide at high concentration (M/100 and M/1000) produced a marked decrease in the amount of paramylum in the cells in long-term experiments. It seems possible that this phenomenon may have been due to the effect of cyanide in increasing activity of amylase, thus resulting in more rapid breakdown of paramylum. The effect of cyanide in stimulating amylase activity under certain conditions has been demonstrated by several workers (Hanes and Barker, 1931; Denny, 1931).

B. Azide

Keilin (1936) studied the inhibition of cellular respiration by sodium azide (NaN_3), and showed that its mode of action was in general similar to that of cyanide. However, he and later workers pointed out certain

TABLE 6

Effect of pH on azide inhibition of respiration of stationary-phase cells

NaN_3 concentration	At pH 5.8		At pH 7.9	
	Inorganic buffer	M/20 acetate	Inorganic buffer	M/20 acetate
	cu. mm. O_2/hour/10^6 cells		*cu. mm. O_2/hour/10^6 cells*	
0 (Control)	1.92	26.51	1.96	26.52
M/1000	2.90		2.31	
M/500		1.26		26.23
Azide inhibition	(Up 51%)	(95%)	(Up 18%)	(1%)

(No cell injury occurred in this experiment.)

interesting differences in the effects of these two substances. In view of these facts a study of azide effects was included in the present investigation.

The relation between pH and azide inhibition of respiration in *Astasia* was investigated (Table 6). In acetate medium, M/500 azide had no effect at pH 7.9, but produced 95 per cent inhibition of respiration at pH 5.8. In inorganic buffer there was no azide inhibition at either pH level.

Somewhat similar relations between pH and azide inhibition have been reported by Keilin (1936), who studied the effect of azide on respiration of yeast cells, and by Armstrong and Fisher (1940), who studied the effect of azide on frequency of the embryonic fish heart. Pointing out that at low pH much of the azide would be in the form of

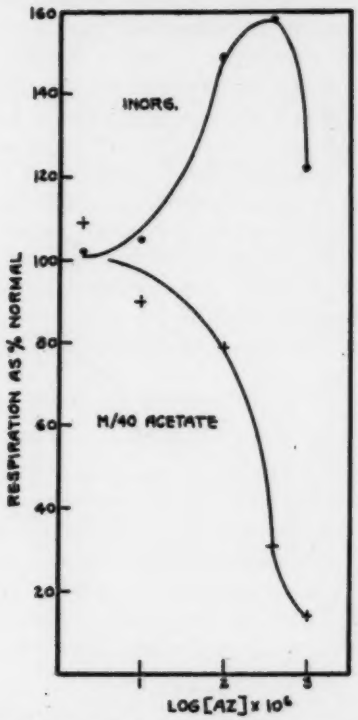

FIG. 4. Effect of different azide concentrations on respiration at pH 5.8. Average values from six two-hour experiments, using cells in logarithmic and stationary phases of growth.

undissociated hydrazoic acid, Armstrong and Fisher concluded that only in this undissociated form could azide enter fish embryo cells. On the other hand, Stannard (1939) found no such pH effect involved in azide inhibition of respiration of active frog muscle.

Experiments were performed at pH 5.8 to determine the toxic level of azide concentration. It was found that in M/250 azide nearly all the cells were injured within two hours (five out of five experiments).

M/500 azide usually injured a large percentage of the cells present (seven out of eight experiments). In M/1000 azide no cell injury was observed in most cases (10 out of 12 experiments). Azide was thus toxic to the cells at much lower concentrations than cyanide.

The effect of azide concentrations between M/500,000 and M/1000 on respiration at pH 5.8 was investigated. Results are shown in Figure 4. In acetate medium, an inhibition curve more or less resembling that produced by cyanide was obtained. But in inorganic buffer, no azide inhibition occurred at any concentration tested, while at certain azide concentrations there was a pronounced increase in respiration over the control values.

Stannard (1939) suggested that in frog muscle there appear to be two distinct respiratory mechanisms: one is characteristic of resting muscle and is only cyanide sensitive; the other functions only in activity, includes the cytochrome system, and is both azide and cyanide sensitive. Similarly, in *Astasia*, it appears that respiration in inorganic medium is mediated through a cyanide sensitive but azide insensitive system; while respiration in acetate takes place through a system which is both azide and cyanide sensitive.

Summary

1. Concentrated cell suspensions of *Astasia klebsii* were prepared from pure cultures. The respiration of these suspensions was measured by the Warburg direct method at 25.2° C., usually at pH 5.8.

2. Cells in inorganic medium showed an early period of declining respiration, followed by an approximately constant period. During the constant period, old (stationary-phase) cells consumed about 1.9 cu. mm. O_2/hour/million cells, and young (logarithmic-phase) cells consumed about 3.8 cu. mm. O_2/hour/million cells.

3. The effects of numerous organic compounds on respiration were tested. Only formate, acetate, propionate, ethyl alcohol, and hexosediphosphate produced a definite increase in oxygen consumption, the greatest increase occurring in acetate.

4. In inorganic medium, respiration was approximately the same between pH 4.5 and pH 7.9. In acetate, respiration was the same at pH 5.8 and pH 7.9, and much lower at pH 4.5.

5. In acetate the respiratory quotient was 1.0; in inorganic medium it was slightly less than 1.0.

6. The presence of cytochrome in the cells was detected by spectroscopic examination.

7. The highest non-toxic concentration of cyanide (M/100) produced about 95 per cent inhibition of respiration in acetate, and a lesser degree of inhibition in inorganic medium. The highest non-toxic concentration of azide (M/1000) inhibited nearly 90 per cent of the respiration in acetate, but had no inhibitory effect on respiration in inorganic medium.

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THE ACCOMMODATION OF SOME MARINE INVERTEBRATES TO REDUCED OSMOTIC PRESSURES

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It has been demonstrated by Pearse (1927), Schlieper (1935), Beadle (1931), Sayles (1935) and others that marine forms will survive dilutions of sea water greater than those encountered in their natural environment for various lengths of time. That this ability will be of importance in determining the degree to which a marine species will colonize areas subject to fluctuations in salinity seems likely, but the precise degree of correlation has been little studied. The work reported here consists of two parts: (1) a survey of the distribution of some marine invertebrates in the Narraguagus River estuary, and (2) an experimental investigation on the tolerance to reduced osmotic pressures of the same forms in the laboratory. The entire study was necessarily confined within the period from February, 1940 through April, 1941. The Narraguagus River estuary is located in the town of Millbridge on the coast of Maine. The ecological study consisted of the location of marine species along the estuary, and a study of the freezing point of the water, water temperature, and type of bottom at selected stations.

The experimental work consisted of (1) the determination of the ability of 14 species to live in diluted sea water, (2) a comparative study of the resistance to diluted sea water of specimens of the same species collected from brackish water and from the open sea, and (3) the influence of diluted sea water upon the weight and oxygen consumption of *Nereis virens*. Finally an attempt has been made to correlate the results of the three investigations in an effort to construct conclusions of ecological importance.

Methods and Observations

The Narraguagus River is relatively large near its mouth and is about 300 feet wide at the town of Millbridge. The distance from the open sea to the head of the tide water is approximately ten miles. The

[1] Based upon a thesis presented by the senior author in partial fulfillment of the requirement for the degree of Master of Arts.

widening bay into which the river empties is hemmed in by numerous islands and mainland points, and at low tide vast areas of exposed mud flats, ledges, and rocky shores furnish an ideal environment for many common marine invertebrates. With such a body of fresh water entering the sea, it seemed reasonable to assume that there would be regular changes in the salt concentration with the tides, and that sessile forms of life would be subjected to rapid changes in the osmotic pressure of the external medium in their natural environment.

Freezing points were measured in a simple cryoscope by means of a Heidenhain thermometer. Samples were collected at 15 stations (see

FIG. 1. Map of the lower portion of the Narraguagus River and of Narraguagus Bay, showing points of collections. Station one is at head tide. Stippled areas represent mud flats exposed at low tide. Depths are at mean low tide. Average tide, 11 feet. After U. S. G. S. Cherryfield and Petit Manan quadrangles.

Fig. 1) in small air-tight jars having a capacity of ten cubic centimeters, and were tested in the laboratory after filtering within 48 hours. As can be seen in Table I, the freezing point values at high tide are lower than those at low tide at all points except station one. In general there is a greater depression of the freezing point towards the open sea, although some irregularities appear because of the contours of the channel.

TABLE I

Results of preliminary investigation of Narraguagus River estuary and location of marine species in this region

Station	Freezing point °C.			Temperature		Marine species
	High tide	Low tide	Water extracted from mud	High tide	Low tide	
1	−.011	−.011	....	21	21	
2	−.021	−.014	....	19	20.5	
3	−.141	−.116	....	18.2	20	*Balanus balanoides*
4	−.948	−.145	....	17.5	19.6	*Grammarus* sp. *Crago septemspinosus* *Nereis virens*
5	−1.006	−.404	....	17.2	19.2	*Mya arenaria* *Mytilus edulis* *Electra pilosa* *Flustrella hispida* *Lepidonotus squamatus* *Spirorbis spirorbis* *Acmaea testudinalis* *Littorina litorea* *Thais lapillus* *Modiolus modiolus*
6	−1.014	−.537	−1.345	....	....	*Balanus balanus* *Balanus eburneus* *Jaera marina*
7	−.978	−.438	−1.702	....	....	
8	....	−1.120	−1.386	....	....	*Sertularia* *Hippothoa hyalina* *Cerebratulus lacteus* *Tegella unicornis*
9	....	−1.212	−1.828	....	....	*Glycera dibranchiata*
10	−1.624	−1.236	−1.622	....	....	*Orchestis platensis* *Nucula proxima*
11	....	−1.615	−1.638	....	....	
12	−1.756	−1.374	....	....	....	*Lineus ruber* *Harmothoe imbricata* *Nephthys caeca* *Clymenella torquata*
13	−1.791	−1.491	....	....	....	*Asterias vulgaris*
14	....	−1.706	−1.717	....	....	*Strongylocentrotus drobachiensis*
15	....	−1.771	....	....	....	
16	−1.791	−1.791	....	....	....	*Metridium dianthus* *Tealia crassicornis*
18	−1.791	−1.791	....	....	....	*Bunodes stella* *Clava leptostyla*

The osmotic pressure of the water confined within the mud flats was investigated, since the animals living in the mud are in contact with this confined water rather than the free surface water. A special type of apparatus (Fig. 2) was designed to extract mud at depths optimal for marine worms without surface contamination. A small portion of each end of the core was discarded and the remainder placed in air-tight jars for transportation to the laboratory. Water was extracted by centrifugation and its freezing point was determined. The tubes were covered during this process to prevent evaporation.

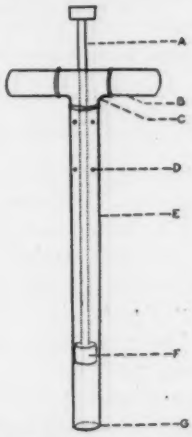

FIG. 2. Mud sampler. *A*. Plunger rod; *B*. Wood handle; *C*. Brass T pipe; *D*. Air expulsion holes; *E*. 1" brass pipe; *F*. Rubber plunger; *G*. Beveled cutting edge.

The freezing points of this "confined" water from all stations show only slight variations, and differ from those of the surface water in the fact that they do not change much in relation to the distance of the station from the open sea. The variations found may be attributed to differences in the physical qualities of the mud at the various stations, and to the proximity of the station to the river channel. It is notable that in every case the osmotic pressure of the confined water is greater than that of the surface water at high tide. This difference is greatest towards the head of the tide water. It is evident that animals burrowing into mud will not be exposed to the rapid fluctuations in osmotic pressure to which animals attached to the surface are exposed.

At each station the marine species present were collected and identified. In Table I each species is listed under the station farthest up

the estuary at which it occurred. It is to be understood that all species were also found at stations nearer the open sea and in greater numbers in these situations. In this work the open sea was considered to be that region at station 16 and beyond where no tidal variation in the freezing point of the water was observed. Particularly noticeable in the low salinity area was the absence of animals belonging to the phyla Porifera, Coelenterata (with the exception of Sertularia), and Echinodermata. However representatives of these phyla were extremely abundant beyond station 15. Another outstanding fact was the size of the animals at station four which were much smaller than similar forms in regions of higher salinity. The worm *Nereis virens* was only two to two and one-half inches long at station four, while it averaged eight inches in the bay proper.

Experiments on Acclimatization to Low Salinity

The experimental animals were brought in from the field and placed in glass dishes of 350 cc. capacity. The dishes were placed in a cooling bath maintained at about 15° C. by means of circulating water. Instead of changing the water in the dishes frequently as other workers have done, air was bubbled continuously through porous "air stones." The entire bath was covered by a glass plate to keep evaporation of the solutions at a minimum. Clean sea water having a freezing point of —1.791° C. was collected from the open sea at station 16. Dilutions were made with fresh water taken from the Narraguagus River above the dam at station one. After ice formed in the river it was supplanted by Orono tap water. The experiments were started in June, 1940, at Millbridge, and were transferred to the University of Maine laboratories in September. Three series of experiments were carried out.

Opportunity for Acclimatization and Survival of Nereis virens in Fresh Water

Twelve worms of the same size and weight were paired and placed in six dishes containing sea water. Worms were carried down to fresh river water as follows: *A*, from sea water directly into fresh water; *B*, from sea water to 50 per cent sea water to fresh water; *C*, through dilution intervals of 25 per cent; *D*, through dilution intervals of 10 per cent; *E*, through dilution intervals of 5 per cent and *F*, through dilution intervals of 1 per cent. All dilution intervals are stated on the basis of the original concentration, and were made either by adding a calculated amount of fresh water, or by changing the entire medium when the capacity of the dish was reached. A period of 24 hours was allowed

for accommodation to each new dilution in groups *A* to *E*. In group *F* the accommodation period was shortened to eight hours, since this period proved long enough to permit adjustment to small alterations in concentration. Control animals were kept in sea water during the entire experiment. The results are given in Table II.

TABLE II

Effects of varying rate of dilution of the external medium on the survival time in fresh water of Nereis virens

Group	Survived 24 hours in each of the following concentrations of sea water	Average survival time in fresh water: hours
A	100 (to fresh water)	6
B	100, 50 (to fresh water)	19
C	100, 75, 50, 25 (to fresh water)	22
D	100, 90, 80 . . . 20, 10 (to fresh water)	32
E	100, 95, 90 . . . 10, 5 (to fresh water)	61
	Survived 8 hours in the following concentrations of sea water	Average survival time in 2% sea water
F	100, 99, 98 . . . 3.2*	72

* Limit of survival in this experiment.

It will be noted that *Nereis* was able to survive in fresh water for longer periods of time as the acclimatization process was made more gradual. Although the animals in this experiment were not weighed, the increased body size and turgidity of the worms due to water intake was very evident in the cases where the dilution interval was large. This turgid condition was less noticeable as the dilution interval became smaller. In group *E* it was not observed until the worms had passed below the 25 per cent sea water level. Subsequently group *F* reached 10 per cent sea water before this condition became apparent. This would indicate that *Nereis* can accommodate to reduced osmotic pressure if the change is gradual enough. It should be noted that the worms in group *F* died in 2 per cent sea water after surviving in that concentration for 72 hours. Work was necessarily suspended at that time so that the survival time in fresh water could not actually be ascertained. At very low salinities frequent changes of the water were necessary, even with a liberal amount of air being supplied. This was due to what seemed to be an excessive expulsion of coelomic fluid which caused putrefaction of the medium. There is a possibility that this fluid was eliminated through the nephridia. The control animals were in good condition when the experiment was discontinued 42 days after it was started.

The Comparative Susceptibility of Fourteen Species to Reduced Osmotic Pressure

Fourteen species representing the phyla Coelenterata, Nemertea, Annelida, Arthropoda, Mollusca and Echinodermata were selected because of their availability in the bay. The apparatus and methods were the same as those described above. Dilutions were made through 1 per cent intervals every eight hours until the animals appeared to have reached their critical point for survival, as indicated by sluggishness and turgidity. The time between changes was then increased to 72 hours. The number of animals in each dish varied between one and 25 depending upon the size. In most cases only two or three were confined in the same dish.

Table III records the results of this investigation. The freezing point values listed are those of the water at low tide at the station where the animals were collected. These areas were selected for collecting specimens because of the numbers available. All animals were in excellent condition when brought to the laboratory with the exception of *Tealia crassicornis* which could not be removed from the rocks without injury to the pedal disc. It should be stated too that it was very difficult to keep *Asterias vulgaris* for any length of time even in natural sea water. The control animals lived very little longer than the experimentals. It is probable that their brief survival was due to other factors. One such factor was nutrition. Even though whole clams and clear chopped clam tissues were placed with them, they failed to eat.

There was considerable variation among individuals of the same species in their abilities to survive in low concentrations of sea water. In a few instances the difference between the minimum and maximum lethal concentrations is high (e.g. *Mya arenaria*), but in most cases the difference is 10 per cent or less. In general it was found that the smaller animals of each species were better able to survive than the larger members. Four *Nereis virens* lived in 1 per cent sea water 52 hours, and three *Gammarus* lived in fresh water for 72 hours. *Glycera dibranchiata* became very swollen and turgid in 50 per cent sea water, and in greater dilutions the integument in certain body regions burst causing extensive loss of blood. *Jaera marina* survived in fresh water for two weeks, and were discarded to make room for other experiments.

On the basis of these results a general phyletic relationship can be established in regard to the ability of animals to survive in reduced salt concentrations. Such a classification stated in order from greatest to

least ability of survival is: Arthropoda, Annelida, Mollusca, Nemertea, Echinodermata and Coelenterata. With the exception of Coelenterata, this order conforms roughly to the degree to which these phyla have succeeded in colonizing fresh water.

TABLE III

The comparative abilities of fourteen species to withstand reduced osmotic pressures arranged in order of decreased resistance

Animal	Freezing point of water in collecting areas	Number used	Per cent sea water withstood −1.791° C.		
			Min.[1]	Max.[2]	Average
Jaera marina	−1.791	25	..	..	0
Gammarus sp.	−1.212	18	0.5	10	2
Nereis virens	−1.120	12	2	2	2
Crago septemspinosus	−0.438	4	3	5	4
Balanus balanoides	−1.212	4	7	13	9
Mytilus edulis	−1.212	8	2	25	9.6
Thais lapillus	−1.212	6	6	24	13
Balanus balanus	−1.212	3	10	18	14
Glycera dibranchiata	−1.212	8	30	38	35
Mya arenaria	−1.120	6	8	55	36
Cerebratulus lacteus	−1.120	6	42	58	47
Asterias vulgaris	−1.791	6	66	84	73
Clava leptostyla	−1.791	2 col.	71	76	74
Tealia crassicornis	−1.791	6	80	90	85

[1] Minimum percentage concentration below which no animals survived for 72 hours.

[2] Maximum percentage concentration above which no animals died within a 72-hour period.

A Comparison of Individuals from the Open Sea and from Brackish Water in Their Resistance to Diluted Sea Water

In this experiment members of four species, collected from areas exposed to the open sea (station 18), were carried through a procedure as described in the preceding section. Their survival was compared with that of brackish water forms collected from station seven. The results of this experiment are shown in the upper part of Table IV with results extracted from Table III added so that an effective comparison can be made. It will be seen readily that in every case the open sea individuals were less resistant to changes in the concentration of sea water than the brackish water forms. The difference is less pronounced in some species, and suggests a higher development of the osmotic regulatory mechanism in these species. The comparison may be made still

more significant by the fact that after open sea specimens of *Mya* had succumbed, brackish water clams introduced into the same concentration of sea water survived and were able to adjust down to a limit of 10 to 8 per cent sea water.

TABLE IV

The comparative abilities of similar open sea and brackish water forms to withstand reduced osmotic pressures

Animals from open sea areas $\Delta = -1.791$	Number used	Dilution per cent sea water		
		Min.	Max.	Average
Nereis virens	6	6	8	7
Gammarus sp.	9	3	16	8
Mytilus edulis	5	39	46	43
Mya arenaria	8	48	58	52
Animals from brackish water $\Delta = -.978$				
Nereis virens	12	2	2	2
Gammarus sp.	18	0.5	10	2
Mytilus edulis	8	2	25	9.6
Mya arenaria	6	8	55	36

Effects of Gradually Reduced Osmotic Pressure on the Weight and Oxygen Consumption of Nereis virens

According to Beadle (1931) *Nereis diversicolor* increases in weight on transfer to dilute sea water and also increases its respiratory rate. Sayles (1935) likewise found an increase in the weight of *Nereis virens* in dilute sea water, but did not determine the respiratory rate. In their experiments the worms were transferred directly to water of low salinity. In the experiment here described five animals were transferred daily through 4 per cent dilution intervals. The Rideal-Stewart modification of the Winkler method was used to determine the amount of dissolved oxygen in the water in closed vessels before the worms were placed in them, and after they were removed. The worms were kept in the closed containers four hours, during which period the apparatus was darkened so as to keep activity at a minimum. Figure 3 shows the results of this experiment. The average weight of the worms shows a slight but consistent decrease even down to 50 per cent sea water. Then there is a marked increase in weight at 28 per cent sea water, but a change to still more gradual dilutions brought the weights down again.

The final weight rise beginning at 16 per cent appeared to be due to the breakdown of the osmoregulatory mechanisms, and was characterized by the intake of large amounts of water which made the animals turgid. Up to this point the animals remained firm with no sign of excess water either in the coelom or the integument.

The results of the oxygen consumption measurements show much more variation between individual worms. This is probably due to differences in the amount of activity. A rather definite trend of the averages is evident, however, and this is correlated with the weight

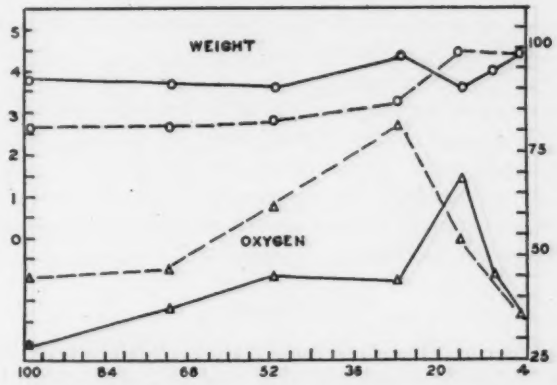

FIG. 3. Effects of diluted sea water on the weight and oxygen consumption of *Nereis virens*. Left hand ordinates represent total weight of five worms in grams; right hand ordinates represent average cu. mm. oxygen per gram per hour. Abscissa; percentage concentration of sea water. Continuous line represents readings as animals were carried from 100 per cent sea water to 4 per cent. Dashed line represents the reverse experiment.

curve. With moderate dilutions the weight remained constant, but oxygen consumption rose 33 per cent. In the experiment described below a corresponding but much sharper decrease in oxygen consumption was found as the worms were returned to normal sea water. We believe that the increased oxygen consumption in dilute sea water represents the expenditure of energy for osmotic work. Below the critical range of 28 to 16 per cent sea water there was a decrease in the amount of oxygen used per gram per hour. Since weight was increasing during this period it is difficult to separate the decrease due to the intake of metabolically inactive water from that which may be expended in osmoregulation.

Weight and Oxygen Consumption of Acclimatized Nereis virens when Gradually Returned to Natural Sea Water

The same animals used in the experiment described above were carried from 4 per cent sea water back to natural sea water, but necessarily at a more rapid rate. Tests of oxygen consumption were made at the same concentrations as in the previous experiment except at 10 per cent sea water. Apparently osmotic regulation is restored as the osmotic pressure of the medium is increased. This is indicated by the sudden decrease in weight accompanied by a marked increase in oxygen consumption up to the critical point at 28 per cent sea water. From that concentration up to full strength sea water there is a direct relationship between the change in weight and the amount of oxygen consumed for, as there is less need for regulation, less energy is expended by the animals. Particularly noticeable at the end of this experiment was the fact that the average weight of the worms had declined 38.5 per cent from the original value. Some of this loss may be due to loss of salts, but since the animals were used experimentally over a period of 94 days, it is probable that other features of an artificial environment may have contributed to this condition.

Discussion

Experimentally all brackish water animals except *Balanus balanoides* were able to survive in media of lower osmotic pressure than those encountered in their natural environment. It may be that the eggs or juvenile stages are less resistant to dilution of the sea water, so that the species fails to invade the less saline areas. It is also possible that the animals avoid water below a certain salt concentration even though they can live in it. In the Narraguagus a complication is found in the presence of accumulations of sawdust from mills located on the river. This is abundant as far south as station four, and undoubtedly has an unfavorable effect on many marine organisms.

According to Adolph (1925) most marine animals are able to live after abrupt change to almost pure fresh water provided that the remaining salts are present in physiological proportions, and gradual dilutions over several days do not materially help the animals to endure pure water. Although he states that some species lived in 1 per cent sea water he names no specific organisms. This is in disagreement with the results of other workers. Pearse (1927) was able to carry few species into solutions weaker than one-fourth sea water, while most of the 18 forms he worked with could not survive in concentrations less

than 50 per cent sea water. Sayles (1935) found the minimum survival concentration for *Nereis virens* to be between 30 and 40 per cent sea water. In these experiments it has been possible to obtain better results than some other workers by keeping the animals at a relatively constant temperature approximating that of their natural environment, maintaining constant aeration, and preparing dilutions with fresh water collected from the Narraguagus River. Dilutions made with distilled water were found to be definitely toxic, while Orono tap water did not give as good results as river water.

Other work bearing on the effect of salinity changes upon respiration appears to be scarce. Beadle (1931) reports that Turussov (1927) found by carbon dioxide measurements with a modified Osterhout apparatus that the respiratory rate of *Nereis diversicolor* increased in hypotonic and decreased in hypertonic sea water. Beadle himself and Schlieper (1935) confirmed these results, but Krogh (1939) states that the small increase in oxygen consumption shown in their calculations does not exceed the limits of error, and therefore cannot be regarded as significant. He admits that energy must be expended for osmotic work, but claims that the amount is too small for demonstration. In view of the unknown efficiency of this process, however, the prediction may not be valid.

Both Beadle and Sayles report that the weights of several *Nereis diversicolor* and *Nereis virens* paralleled one another for a time, but that some would soon decrease in weight at the same time that others were increasing. In many cases those animals that continued to increase in weight became turgid with a resulting rupture of the body wall allowing considerable loss of body fluid. The weights of *Nereis virens* in the experiments here reported showed very little variation, and then only in the extremely low salinities, while no animals were observed to become swollen to the bursting point. *Glycera dibranchiata*, on the other hand, took on large amounts of water in concentrations of 50 per cent sea water and in 39 per cent sea water the integument burst with considerable loss of blood and internal fluids.

A comparison of the relative ability of the three species of *Nereis* which have been tested is difficult because of differences in technique. *Nereis virens* appears to use less oxygen per gram weight than do the other two species, but on the basis of surface area the amounts consumed are about the same.

Summary

1. The distribution of 14 species of invertebrates in the Narraguagus Bay and River was compared with the osmotic pressure (indirectly de-

termined by means of freezing points) of the water at a series of stations ranging from the open sea to the head of the tide.

2. The osmotic pressure of the water confined in the mud flats was found to be almost constant at high and low tides in contrast with the marked tidal variations in surface water.

3. Typical marine animals were found in an environment having a freezing point of — .404° C., or the equivalent of 19 per cent sea water.

4. The survival of *Nereis virens* in fresh water was extended as the process of acclimatization was made more and more gradual.

5. Tests on 14 species showed that all but one were able to survive experimental exposure to reduced osmotic pressure below that found in their natural environment.

6. Members of a species collected from water of low salinity were more resistant to reduced osmotic pressure than individuals collected from the open sea.

7. *Nereis virens* was able to accommodate without marked change of weight down to a concentration of 16 per cent sea water. At this point a rapid increase in weight occurred. The dilution process above 16 per cent was accompanied by an increased oxygen consumption. Below 16 per cent sea water oxygen consumption decreased.

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INCREASE OF CORTICAL CALCIUM WITH AGE IN THE CELLS OF ELODEA CANADENSIS[1]

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The suggestion has been made frequently that senescence results from progressive accumulation in the cell of materials which either are toxic or obstruct metabolism (Jickeli, 1902; Montgomery, 1906; Child, 1915; Seifritz, 1936; Heilbrunn, 1937). Benedict (1915) formulated the hypothesis that senescence is a result of a decrease in the permeability of cells. Both of these causes of senescence may be the result of an increase with age in the calcium content of the cell membrane, for calcium decreases the permeability of cells to at least some classes of substances (see Heilbrunn, 1937, for literature review). Thus it is conceivable that an increase in the calcium content of the cell cortex could decrease the cellular permeability to its toxic metabolic waste products as well as other substances. A gradual accumulation of these substances in the cell may be a consequence of these preceding changes, and singly or in combination may produce the degenerative changes of senescence. In view of the universality of senescence the author inclines towards the hypothesis that the single or series of related cellular changes sketched above is its universal cause. He proposes therefore to present a series of studies on the possible localized increase of calcium with age in a variety of organisms, and its consequences. It should be noted that Molisch (1938) suggested a relation between the deposition of calcium with age in cell membranes of plants and a decrease in the permeability of cells.

Calcium Changes With Age

Increase of calcium with age is one of the commonest features of the aging process. The calcium content of many human tissues and organs has been shown to be greater in old individuals than in young individuals

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(Burger and Schlomka, 1927; Delbet and Bretau, 1930; Hesse, 1934; Sorsby, Wilcox and Ham, 1935; and Simms and Stolman, 1937).

The work of Novi (1913) on other mammals is in agreement with the findings in human material; he reports that the calcium content of the brain of the guinea pig at birth is very low, but toward the end of the life span it increases markedly. Similarly, Lansing (unpublished observations) found a progressive increase in the calcium content of the rat brain with age. On the other hand, Novi (1913) found that the calcium content of the dog brain is very high in the fetus, is very low in the young animal and finally returns to the high level of the fetus in the old dog. Sorsby, Wilcox and Ham (1935) reported that the amount of calcium in the sclera of the cat eye decreases from birth to adult life, then increases with old age. Lastly, Cahane (1927) concluded that the amount of calcium per gram of fresh muscle of the guinea pig, dog, cat and rabbit decreases with age. It is apparent from Cahane's data, however, that the majority of animals referred to as "old" were actually either immature or young adult animals.

The preceding studies, with the exception of the one by Cahane (1927), demonstrate that the calcium content of mammalian tissues increases after maturity. It is also apparent that the calcium content of fetal tissues may be very high, and that the calcium content decreases in some animals during the period of immaturity.

As far as the writer has been able to determine, there have been no studies of calcium changes with age in plants or invertebrates. The observation that the amount of insoluble salts of calcium increases with age in plants is a common one but has not been put on a quantitative basis. Molisch (1938) points out that calcium oxalate and carbonate are precipitated about the cell walls of plants and increase with age. He suggests the possibility that calcium increases with age in the cell membrane but offers no evidence to support this view. The writer has undertaken a study of calcium changes with age in plants and the invertebrates. This paper deals with the water plant, *Elodea;* a later paper will deal with the calcium changes with age in the invertebrates *Euchlanis,* a rotifer, and *Phagocata,* a planarian.

Mazia and Clark (1936) studying the effects of various stimulating agents on *Elodea* leaves found that upon stimulation calcium is released from the cortex of the cell and combines with the soluble oxalates in the central cell vacuole to form the insoluble crystals of calcium oxalate. The technique of Mazia and Clark suggests a means of determining the amount of calcium in the cortex of *Elodea* cells of varying ages. The following experiments were undertaken to investigate this possibility.

Materials and Methods

Freshly cut stalks of *Elodea canadensis* (*Anachris canadensis*) grown in a glass aquarium at room temperature were employed in all the experiments. The stalks used were a minimum of six inches in length and a maximum of ten inches.

The relative age of an *Elodea* leaf is easy to determine by the position of the leaf on the stalk. Young leaves are located at the apex of the stalk and old leaves are located at the base of the stalk.

The technique used for the study of the calcium content of *Elodea* cells was based upon the observation by Mazia and Clark (1936) that stimulation of the *Elodea* cell results in the combination of the calcium in the cortex of the cell with the oxalates in the vacuole to form insoluble crystals of calcium oxalate. Stimulation was electrical in the present experiments.

Leaves were removed for analysis from various parts of the stalk and were placed on glass slides. Two 1½ volt dry cells were connected in series with an induction coil set for interrupted current. Platinum electrodes were placed directly on the moist leaf and the electrodes were reversed immediately upon the expiration of one half of the total time of stimulation which was two minutes.

Tests were conducted to determine the amount of stimulation required to produce maximal formation of calcium oxalate crystals in the cells. It was found that stimulation for two minutes was adequate to produce the maximal effect in all cases. A young leaf was stimulated for 40 seconds. Thirty-five crystals per cell were formed and subsequent stimulation produced no increase in the number of crystals. Stimulation of an old leaf for 40 seconds produced 15 to 20 crystals, stimulation for 60 seconds produced 30 to 40 crystals, while stimulation for 80 seconds produced 50 to 55 crystals per cell. Further stimulation produced no increase in the number of crystals formed. This strongly suggests that old cells are less susceptible to stimulation than young cells, or rather that they are less irritable; however, no studies were conducted to verify this point.

The description of the calcium oxalate crystals furnished by Mazia and Clark (1936) was used as a basis of identification of the monoclinic and tetragonal crystals found in these experiments. Five small and large tetragonal and large monoclinic crystals, and 46 small monoclinic crystals (in apical and basal leaves) were selected at random for measurement. The lengths and widths were measured by means of a micrometer ocular and the depths by the calibrated fine adjustment screw of the microscope. The depth measurements involve an error of $\pm$ 0.5

micron. Small monoclinics average 0.9 ± 0.08 micron in width, 2.5 ± 0.09 micron in length and 1.0 micron in depth. Small tetragonal crystals average 3.3 ± 0.08 micra in width, 3.3 ± 0.05 micra in length and measure 2.0 micra from apex to apex of the crystal. Large tetragonal crystals average 6.8 ± 0.21 micra in width, 7.2 ± 0.00 micra in length and 4.5 micra from apex to apex of the crystal. Large monoclinic crystals average 3.3 ± 0.25 micra in width, 6.2 ± 0.32 micra in length and 3.0 micra in depth.

The volumes of the various types of crystals were computed on the basis of the above measurements. As the monoclinic crystals are rectangular parallelopipeds, the product of the three linear dimensions of the monoclinic crystals was employed for the volume calculation. Since the tetragonal crystal is essentially two pyramids whose bases are in juxtaposition the formula for computing its volume is two times the area of the base times the height of the pyramid divided by three, i.e., twice the volume of a pyramid. Thus the average volume of the small monoclinic crystals is 2.2 ± 0.33 cubic micra, small tetragonal is 21.5 ± 0.7 cubic micra, large tetragonal is 222.3 ± 13.4 cubic micra and large tetragonal crystal is 62.6 ± 7.0 cubic micra. The calcium content of the cells studied was determined by making counts of the number of crystals present after stimulation and computing the total volume of crystalline material.

Since the amount of crystalline material formed in these experiments could possibly be correlated with the amount of cell surface present as well as the concentration of calcium in the cell cortex, it was necessary to determine the total amount of surface for the various cells.

Measurements with a micrometer ocular were made of six cells in the tip region of young and of old leaves. The sum of the areas of the surfaces of the cell was used in the computation of the total cell surface. The total surface of the mean apical cell in apical leaves was 2919 square micra and of the mean apical cell of basal leaves was 3139 square micra. Thus, the total cell surface of apical cells of basal leaves was only seven per cent greater than that of apical cells of apical leaves.

Quantitative determinations of the oxalate content of apical regions of young leaves were made. For each determination 75 leaves were used; the anterior fifth of each leaf was cut off and put in a porcelain crucible containing distilled water. The material was thoroughly crushed and the supernatant fluid decanted. The residue was washed several times with distilled water and the washings added to the material previously removed. The remaining pulp was filtered and washed, the filtrate again being added to the previous wash solutions. The solution was rendered alkaline with NH_4OH and 2 cc. N/3 $CaCl_2$ was added in

order to obtain complete precipitation of the oxalate. The solution was then filtered and the residue dissolved in 15 cc. of hot H_2SO_4 (1:5). The filter paper was washed with an additional 15 cc. of H_2SO_4 at 70° C., and the filtrate was added to the solution previously obtained. The solution was kept at 70° C. and titrated against 0.01 N $KMnO_4$. Two experiments on tip regions gave the following results: 0.0176 and 0.0107 gm. oxalic acid per gram of leaf material.

Results

The cells in *Elodea* leaves are arranged in longitudinal rows which may be readily traced along the length of the leaf. In five series of experiments counts were made of the number of crystals formed after stimulation in the tip regions of leaves at different positions on the stalk. Only the upper surface of the leaf was studied, and rows of cells ap-

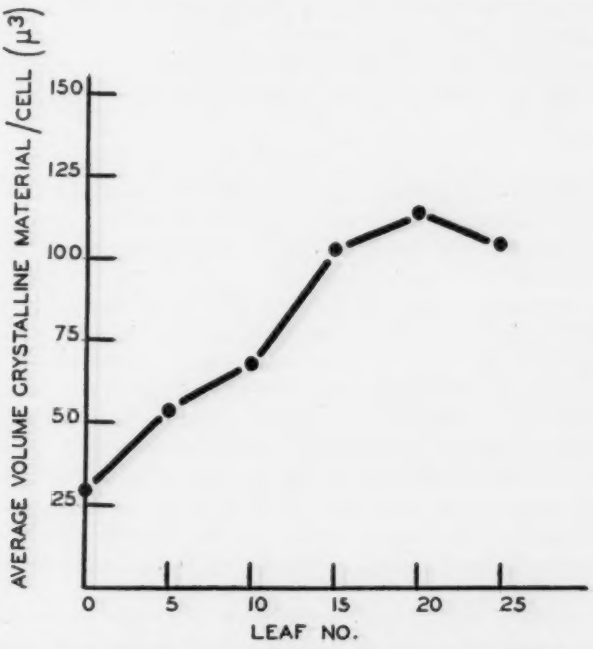

Fig. 1. Graph showing the average volume of crystalline material per cell formed after stimulation of *Elodea* leaves of different ages. The tip leaves are represented by 0, basal leaves by 25.

proximately halfway between the midrib and the edge of the leaf were selected for analysis. Beginning at the apex of the stalk every fifth leaf was removed and stimulated. Only the first 20 cells from the apex of the leaf were used and every fourth cell was counted so that for each leaf there were five cells counted. The computed data for the total amount of crystalline material in these experiments are shown graphically in Figure 1. Using the volume data mentioned previously it was determined that the various leaves contained the following amounts of crystalline material:

Apical leaf	29.5	cubic	micra
Fifth leaf	55.7	"	"
Tenth leaf	67.8	"	"
Fifteenth leaf	102.3	"	"
Twentieth leaf	114.0	"	"
Twenty-fifth leaf	104.3	"	"

In contrast with the group of apical leaves studied in which no tetragonal crystals were found, one large tetragonal crystal was noted in the fifth leaf group, one small tetragonal crystal in the tenth and fifteenth leaf groups, one large and five small tetragonals in the twentieth leaf group, and seven small tetragonal crystals in the twenty-fifth leaf group.

Discussion

Mazia and Clark (1936) state that stimulation of *Elodea* cells causes a release of calcium from the cortex into the cell interior where the free calcium combines with the soluble oxalates in the central cell vacuole to form the crystals of calcium oxalate.

The experiments reported by the writer show clearly that there is an increase with age in the amount of calcium oxalate formed after stimulation of the cells of *Elodea*, and it may be concluded that the cell cortex of old *Elodea* cells contains more calcium than young *Elodea* cells. The quantitative analyses of the oxalate content of young leaves conducted by the writer (see section on methods) show that oxalates are present as at least 1 per cent of the total wet weight of leaf material. As this concentration of oxalate is far in excess of the concentration of calcium in living tissues, an increase with age in the amount of oxalates would have no effect on the amount of calcium oxalate formed after stimulation.

The question then arises whether there is actually an increase with age in the amount of calcium per unit of cell surface or whether there is an increase in cell surface which would account for the additional calcium in old cells. The cell measurements referred to earlier show a

7 per cent difference in cell surface between apical cells from tip and basal leaves. Obviously, a 7 per cent increase in cell surface is not adequate to account for the 300 per cent increase in the amount of crystalline material observed in the present experiments, and it may be concluded that the concentration of calcium per unit of cell surface increases with age in the cortex of *Elodea* leaf cells.

The results obtained in these experiments demonstrate that calcium increases with age in plants as well as mammals. The amount of cortical calcium per unit of plant cell surface increases with age.

Summary

By means of electrical stimulation of the leaves of *Elodea canadensis*, insoluble crystals of calcium oxalate were formed in the central vacuole of cells of different ages. There was an increase with age in the amount of crystalline material formed; moreover, the amount of cortical calcium per unit of cell surface increased with age.

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INCREASE OF CORTICAL CALCIUM WITH AGE IN THE CELLS OF A ROTIFER, EUCHLANIS DILATATA, A PLANARIAN, PHAGOCATA SP., AND A TOAD, BUFO FOWLERI, AS SHOWN BY THE MICROINCINERATION TECHNIQUE [1]

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In the preceding study the writer (1942) demonstrated that the amount of calcium in the cortex of the leaf cells of *Elodea* increases with age. The following studies were conducted in order to discover whether a similar increase in calcium content occurs in representative animal cells.

Material and Methods

Material for analysis was chosen so as to include both multiplying and non-multiplying cells of diverse organisms. The rotifer, *Euchlanis dilatata,* and the gastrocnemius muscle of the toad, *Bufo fowleri,* were selected to provide examples of non-multiplying cells; the planarian, *Phagocata* sp., provides within the same animal both multiplying and non-multiplying cells.

Euchlanis dilatata

The stock of this rotifer was developed from a single animal found in a pond near Bloomington, Indiana. The rotifers were cultured in the laboratory on pyrex depression slides containing two drops of culture fluid (Sonneborn, 1936). A number of newly-hatched rotifers were isolated and 40 of these animals were removed and fixed for histochemical analysis. The remaining animals were cultured on the depression slides throughout the entire life span. The animals were transferred daily to fresh depressions and samples of 20 animals were taken each day for chemical analysis. The last sample was taken at the end of the fourth day at which time the rotifers manifested the characteristic

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changes of senescence. The maximum age of *Euchlanis dilatata* when grown at 30° C. is four to five days. This was determined in a number of experiments on the normal life cycle (unpublished data).

Phagocata sp.

Several hundred of the multipharyngeal planarian, *Phagocata* sp., were collected from a spring near Bloomington, Indiana, and brought into the laboratory. The planaria were examined with a dissecting microscope and measured while fully extended. The smallest animals found were 3 to 4 mm. in length; 25 of these small planaria were selected for chemical analysis. A second group of 25 animals 5 mm. in length and markedly broader than the preceding group of animals, was selected for analysis to represent the planaria of medium size, since these were most abundantly found. The largest animals were 10 mm. in length; 25 of these were selected at random for chemical analysis. On the basis of size it was assumed that the planarians 3 to 4 mm. in length were the youngest of the three groups and that the planarians measuring 10 mm. in length were the oldest. This conclusion was substantiated by a comparison of the degree of pigmentation of the animals. The smallest animals possessed the light gray pigmentation of young planaria while the large animals were a dense black, a characteristic of adult *Phagocata*.

Bufo fowleri

A collection of toads was made during the third week of August in the vicinity of Greenwood Lake, Indiana. All the animals were gathered from a relatively small area in order to reduce environmental variations to a minimum.

Six toads measuring 5.6 to 6.9 cm. in length (fully extended) were selected for analysis and pithed. The gastrocnemius muscles were carefully excised and were immediately put into absolute alcohol-formalin fixative. Six toads measuring 13.6 to 15.7 cm. were pithed and the gastrocnemius muscles excised and fixed as were the previous group. On the basis of the length measurements described above and the growth data of Hamilton (1934) whose work was done on the closely related toad, *Bufo americanus americanus* Holbrook, it was concluded that the toads measuring 5.6 to 6.9 cm. were four to five months old and that the toads measuring 13.6 to 15.7 cm. were two or more years old.

Microincineration Technique

The technique of microincineration employed in these experiments is essentially the same as that of Scott (1933a). Satisfactory results were

obtained by the author (1938) with this technique in an analysis of the localization of calcium in *Paramecium caudatum*. The experimental material was fixed in absolute alcohol-formalin (9 pts. to 1 pt.), dehydrated, cleared in xylol, and imbedded in paraffin. The rotifers and planaria were sectioned at six micra and the gastrocnemius muscle of the toad was sectioned at eight micra. The sections were mounted on clean glass slides and placed in an electric furnace. As recommended by Scott, the initial temperature increase was effected slowly, 20 minutes being required to obtain a temperature of 100° C. The temperature was then raised to and kept at 600° C. for 30 minutes which was found to be adequate for complete volatilization of the organic substances. The slides were cooled very slowly and placed without cover glasses in clean slide boxes to make possible future chemical analysis of the incinerated material. No attempt was made to analyze other elements besides calcium although some measure of the iron content of the preparations could be obtained from inspection of the untreated slides. A reddish-yellow color in the untreated incinerated preparations indicated the presence of iron and the intensity of this color could be used as an index of the iron content.

Calcium was identified chiefly by means of the alizarin reaction. (Saturated aqueous sodium alizarin sulfonate reacts with calcium to form the red precipitate of calcium alizarinate.) Occasional checks on the alizarin reactions were made by means of the delicate gypsum reaction. The reagents used in this reaction were one microdrop of 0.1 N HCl followed by one microdrop of 0.1 N H_2SO_4 (Scott). The presence of calcium was indicated by the formation of needle-like crystals of calcium sulfate. Since there was a marked diffusion of salts following the HCl treatment, precise localization of calcium could not be effected by this reaction, and for that purpose the alizarin reaction was depended upon completely. There was no piling up of the granules of ash in the incinerated sections since the material was cut at six and eight micra; that is, the incinerated preparations were but one granule deep. Consequently, an increase in the number of granules which stain red after the alizarin treatment, and thus an increase in the intensity of the staining reaction could be taken to mean that the amount of calcium had increased. Similarly, an increase in the number of calcium sulfate crystals formed, using the alternative test, was taken as an index of an increase in the calcium content.

Slides of the various organisms were stained with Ehrlich's haematoxylin, and these preparations were compared with the incinerated material for the purpose of identifying structures. As will be brought

out later in the paper, material stained with Ehrlich's haematoxylin may be used in direct determinations of the calcium content of the tissues with the intensity of staining as an index of the calcium content.

Results

Bufo fowleri

The gastrocnemius muscle of the toad when incinerated furnishes a picture very similar to that obtained by Scott (1932) in his microincineration studies on striated muscle. The sarcolemma shows in young fibers as a very fine, white line of ash. The residual ash of the sarcolemma in many preparations is so slight as to be almost invisible. The ash of the sarcolemma in old muscle fibers is strikingly different from that of young tissue. Here the line of white ash is glaring and heavy. The striations show in young and old muscle fibers as parallel rows of discrete, small white granules. However, the granules in the striations of old muscle fibers are markedly larger than those in young muscle fibers. As observed by Scott, the isotropic bands or the J bands appear to be ash free. In these preparations no trace was found of the intermediate bands of the J striation, or the Z band, although Scott reports observing the ash skeleton of the Z striation in "favorable" material.

Tests with sodium alizarin sulfonate demonstrate that the nuclei of young and old muscle fibers are both rich in calcium. The large amounts of this cation present in the nuclei rendered it impossible to estimate quantitative differences between young and old muscle fibers. The sarcolemma of young muscle fibers after alizarin treatment shows the red color characteristic of calcium alizarinate and tests with the gypsum reaction reveal the needle-like crystals of calcium sulfate. Contrary to previous experience with other tissues it was possible to use the gypsum reaction for quantitative estimations of calcium because of the large size of the muscle fibers. The sarcolemma of old muscle fibers gives the same positive tests for calcium but consistently demonstrates a marked increase in calcium content, as indicated by an increase in the staining intensity with alizarin and an increase in the number of crystals formed in the gypsum reaction. Similarly, the striations of old muscles show a marked increase in the concentration of calcium over the striations of young muscle fibers. The nuclei of both young and old muscle fibers contain varying amounts of iron as indicated by the presence of reddish-yellow granules. Iron is also present in the striations of both young and old fibers and there appears to be an increase in the iron content of the striations with age.

Phagocata sp.

Incinerated preparations of this multipharyngeal planarian show an interesting variation in the total ash content of the various tissues. The epithelial lining of the body is richest in inorganics. The pharynges show a markedly low total inorganic content in contrast to the high salt content of the epithelium (Fig. 1). The ash in the pharynges is a dull bluish-white and is uniformly scant in the various tissue layers of these organs. However, the cell membranes of the cells lining the lumina of the pharynges contain considerably more ash than other regions of these organs. Throughout all the tissues and organs that were examined

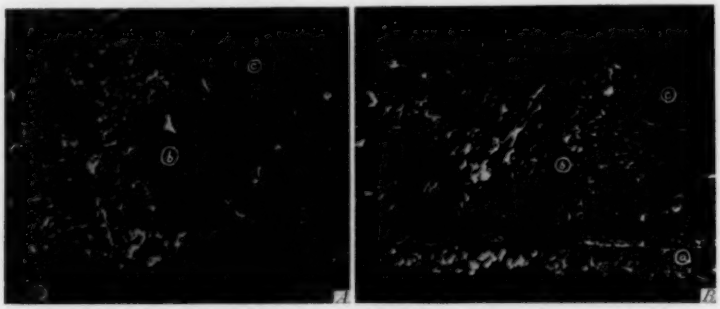

FIG. 1. Photomicrographs of incinerated preparations of *Phagocata*. Sections cut at 6 micra. (*A*) Young animal showing relatively large amounts of ash in the interstitial cells and small amounts of ash in the pharynges. (*B*) Old animal showing the increase in the ash content of the interstitial cells and the epithelial cells. Ash outline of some of the cells of the pharynges can be distinguished. Epithelium (*a*), Interstitial cells (*b*), Pharynx (*c*). Eastman "Contrast Process Panchromatic" film; 500-watt projection lamp; 15-second exposure, 200 magnification.

there was an increase in the total amount of ash with age. The basal membrane of the epithelial lining of the body in young animals is very thin and difficult to detect, but in old specimens the basal membrane is a glaring white line of ash. The increase in total ash content of the cell membranes of epithelial, interstitial (Fig. 1), and nerve cells is apparent. There appear to be no detectable changes in the total ash content of the rhabdites or the nuclei in the epithelial layer with age. This may be due to the fact that even young preparations are very rich in inorganics in these structures and an increase would be difficult to detect. The tissue layers of the pharynges, which as previously stated contain relatively little ash, show little or no increase with age in total ash content (Fig. 1).

Application of the chemical tests for calcium demonstrates that calcium increases with age in all the cells studied. The epithelial layer

is rich in calcium in young as well as old specimens, but there is a marked increase in the calcium content of the cell membranes of the epithelial cells and basal membrane with age. There is an increase in the calcium content of the nuclei and cell membranes of the cells of the pharynges with age, but the change here is far less than it is in the epithelial layer. Repeated tests for calcium in the cells of the pharynges confirm this slight change with age in the calcium content. The cilia of the lining of the pharynges are apparent in most of the preparations but no changes in their calcium content could be observed. The most marked increase in calcium content with age in the cells of the pharynges occurs in the cell membranes of the cells lining the lumina. A pronounced increase in the calcium content of the interstitial and nerve cells was observed. Calcium increases with age in the nuclei, cytoplasm, and particularly the cell membranes of these cells.

Examination of the untreated incinerated slides indicates that the epithelial and interstitial cells, and the rhabdites are richest in iron content. The epithelial and nerve cells show no increase in iron content with age. The interstitial cells reveal an inconsistent fluctuation in iron content with age. The cytoplasm of these cells, in some preparations, shows an increase in iron content with age while in other preparations there was no perceptible difference in the iron content of young and old specimens.

The picture obtained in the animals of medium size was very similar to that found in the group of large planaria. However, the concentration of calcium in the various cells of the large planaria was somewhat greater than that found in the cells of the medium group.

Euchlanis dilatata

Incinerated preparations of this rotifer manifest the same age changes that were observed in *Phagocata*. There was no significant difference in the amount of total ash in the cells of the one- and two-day-old specimens. However, the three-day-old rotifers contained much more ash than did the younger material, and the amount of total ash increased still further in the four-day-old material.

Tests with sodium alizarin sulfonate and the gypsum reaction showed that the increase in the calcium content of the rotifers paralleled the total ash increase. Thus, the first indication of an increase in calcium content was observed in the three-day-old material and a further increase was found in the four-day-old rotifers. Difficulty was experienced in identifying the skeletons of the various cells in this rotifer and only the cells of the brain, gut, and ovary showed clearly in the incinerated prep-

arations. This may be due to the fact that the animals contract somewhat during the fixation process. Tests indicated that calcium increased markedly in the nuclei, cytoplasm and cell membranes of these cells in the three- and four-day-old rotifers. The cell membranes of the one- and two-day-old rotifers showed as thin rings delicately tinted with a red color after they were stained with alizarin. The cell membranes of the three- and four-day-old rotifer material showed as heavy rings of ash which were stained a deep red with the alizarin.

The iron content of the cells of *Euchlanis* appeared to vary somewhat with age, but the iron changes were not as consistent as in *Phagocata*. There was a distinct increase in the iron content of some of the four-day-old specimens over the younger material. On the other hand, specimens were found in which the iron content was higher in the two- and three-day-old material than it was in the four-day-old material. The iron was found to vary most in the nuclei and cell membranes.

Ehrlich's Haematoxylin

It was earlier stated that control slides were prepared which were stained with Ehrlich's haematoxylin. Routine checks made with these slides on incinerated preparations revealed a striking relationship between the intensity of haematoxylin staining of the various cell types and the calcium distribution and content of these cells. In agreement with Scott (1933) it was found that structures rich in calcium as determined by microincineration invariably stained a dark purple with the haematoxylin, and structures with a low calcium content stained but lightly. Thus, the cells of the pharynges of *Phagocata* were delicately stained with haematoxylin in contrast to the other body tissues of *Phagocata* which stained heavily. As observed previously, the pharynges were very low in calcium content. The basal membrane of the epithelial layer of *Phagocata,* which analysis for calcium following microincineration proved to be rich in calcium, stained a dense purple with the haematoxylin. Similarly, the cell membranes of the epithelial and interstitial cells which were demonstrated to be rich in calcium also stained very heavily with the haematoxylin. A comparison of stained slides of young and old planarian and toad material revealed that the old material under the same staining conditions invariably stained more heavily than did the young material, and that the regions that stained more heavily corresponded to those regions which manifest an increase in calcium content with age. This relation between the intensity of staining with Ehrlich's haematoxylin and the calcium content has been frequently observed by the author in various types of material.

The use of haematoxylin as an indicator for calcium has been criticized by Lison (1936) in his excellent book on histochemical methods. He states that the color reaction between haematoxylin and calcium is dependent upon the presence of aluminum, chromium or gold in the fixatives used. However, despite the fact that the reaction is indirect, the close correlation between the calcium content of a structure and the intensity of haematoxylin staining of that structure justifies the use of this simple staining procedure for the identification and localization of calcium in cells and tissues.

Conclusions

The present studies demonstrate that a calcium increase with age, observed in the cortex of *Elodea* cells (Lansing, 1942), occurs similarly in the cells of two invertebrates and in the muscle tissue of a vertebrate. The widespread occurrence of this localized calcium increase with age suggests that it is a general characteristic of the aging process.

Further development of the relation between calcium and aging from both a descriptive and experimental point of view will require an objective quantitative method for the determination and localization of calcium in cells. Further, since it has been demonstrated that calcium increases with age in many tissues and organs of mammals (Lansing, 1942), it would be pertinent to determine whether the calcium increase with age in mammals also takes place in the cell membranes.

Summary

A rotifer, *Euchlanis dilatata,* a planarian, *Phagocata* sp., and toad, *Bufo fowleri,* were selected for an analysis of calcium localization and of possible calcium increase with age. Samples of animals of different ages were sectioned, incinerated and examined with a darkfield microscope. Calcium was identified by means of sodium alizarin sulfonate and the gypsum reaction. It was demonstrated that calcium increased with age in the cell membranes of all the cells studied. Calcium also increased with age in the nuclei and cytoplasm of nerve and interstitial cells of *Phagocata* and the cells of *Euchlanis.* Because of the large amounts of calcium present in the nuclei it was difficult to determine the degree of calcium increase.

The iron content of the various cells appeared to vary considerably but inconsistently with age.

Control slides stained with Ehrlich's haematoxylin showed a correlation between the intensity of staining of structures and the calcium content of those structures. The suggestion was made that Ehrlich's haematoxylin may be used as an indicator for bound calcium in cells.

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THE HYPOPHYSIS OF THE BROAD-BILLED SWORDFISH, XIPHIAS GLADIUS L.

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The life history of the broad-billed swordfish (*Xiphias gladius L.*) is unknown, despite the investigations of marine biologists for a considerable period (Goode, 1873; Fish, 1926). Information concerning the habits of the adult fish, the location of its breeding grounds, or the occurrence of sexually mature swordfish is completely lacking. Therefore, a knowledge of the reproductive condition of the swordfish in all parts of its extensive range would be helpful in locating its breeding areas. The purpose of this paper is to determine primarily the aspect of the hypophysis of those swordfish which occur in southern New England waters. Their state of ovarian development has been described (Lee, 1942). It is hoped that these studies which have established the reproductive condition of those swordfish which are taken in this region, will be extended to include fish from all parts of its range, eventually locating its breeding grounds by this means.

The study of the hypophysis of the swordfish captured off the southern New England coastline was begun in the summer of 1940. Twenty-three pituitaries were obtained during this season, but the sex of the fish from which they were taken was undetermined. Therefore, the work was repeated in the summer of 1941 on glands from 13 swordfish, all of which proved to be female. Their pituitary glands were identical with those obtained during 1940.

Methods

Dawson's and Friedgood's (1938) method of preparing the pituitaries of the rabbit and cat for study was used throughout this examination of the swordfish gland. This method was used successfully by Scruggs (1939) in his studies of the hypophyses of 16 different fish. The glands were fixed for 48 hours in ten parts of 0.1 per cent NaCl solution supersaturated with $HgCl_2$, to which one part of formalin was added just prior to use. Serial sections were cut at three to four micra.[1]

* Contribution No. 317.

Owing to the conditions of obtaining the pituitaries, an interval of from four to six hours often occurred between the capture of the fish and the fixation of the gland. However, all classes of cells appeared maintained, cytoplasmic granules were preserved, the vacuolar membranes were intact and negative images of the Golgi apparatus were readily found. The fixation compared favorably with that obtained for other fish pituitaries fixed immediately by the same method (Scruggs, 1939) and with one swordfish pituitary fixed less than 40 minutes after capture of the fish. The relatively good preservation of the material was probably due to the fact that the heads were removed and packed in ice below decks upon capture of the swordfish.

Results

The hypophysis lies beneath the infundibular region of the brain in a deep sella turcica, which is lined with periosteum and with that portion of the meninx that descends to form a tightly fitting sheath about the gland. As in higher organisms, the dorsal surface of the gland is covered by a diaphragm of the meninx, which is reflected to form a collar of connective tissue between the hypothalamus and the pituitary. There is no saccus vasculosus, nor any structure which may be likened to it, in the swordfish.

The pituitary gland is relatively large with respect to the brain. In four specimens chosen at random, the gland averaged 0.13 cc. in volume and weighed 0.08 gm., wet weight. In shape it resembles a blunt tetrahedron with its base pressed closely against the infundibulum. One surface faces anteriorly and the other two extend postero-medially to form the posterior margin of the gland. From the corners and apex of the hypophysis, extensions of the meninx adhere to or fuse with the periosteum, binding the gland tightly into the sella.

Sagittal and frontal sections of the pituitary reveal that the gland is divided into the four usual regions described by Stendell (1914) and other investigators of the teleost pituitary gland: the pars nervosa, the pars intermedia, the pars anterior, and the Übergangsteil; but the topographical distribution of these regions varies from the customary arrangement.

[1] The brain of the broad-billed swordfish is exceptionally small in relation to the size of the body. The average brain weight was 1.9 gm. and the average length was 3.5 cm. in four specimens chosen at random, whose average weight was 160 kg. and whose average total length was 2.8 m.

The pars nervosa

There is no discrete stalk of nervous tissue connecting the hypophysis to the brain. A slender tongue of the infundibular recess descends through the ventral medial sulcus, then expands laterally to form a small secondary cavity of an inverted mushroom shape. From the thin ventral wall of this cavity, which lies directly in contact with the dorsal surface of the hypophysis, the pars nervosa penetrates the pars glandularis. The pars nervosa appears primarily as a thick stalk of nervous tissue extending into the center of the hypophysis. Here it arborizes completely into a tree-like structure which is entirely surrounded by the pars intermedia. Accessory penetrations of tissue from the infundibular region project into the gland, peripherally to the main nervous stalk. The exact nature of these secondary penetrations is unknown, but they appear to be combinations of connective tissue and blood vessels from the meningeal diaphragm, with slender trabeculae of nervous tissue which descend directly from the infundibulum. The pars nervosa possesses the fibrous nature reported by Bock (1928), Charipper (1936), and others for this region of the teleost pituitary gland. The nuclei of connective tissue cells are common; but the chromatic granules, or Herring bodies, such as were found by Scruggs (1942) in the pars nervosa of the goldfish and carp, were not found in this portion of the swordfish pituitary.

The cells in the pars anterior of the mammalian pituitary are described as "acidophiles," "basophiles," or "chromophobes," depending upon whether they assume the so-called "acid" stains such as eosin, orange G, or azocarmine, the so-called "basic" stains such as methyl blue, or assume no stain whatever. This terminology has been transferred to cells within the three subdivisions of the teleost pituitary gland, which react in apparently similar fashion, by Matthews (1936), Scruggs (1939), Kerr (1940) and other investigators. A like procedure has been adopted in the description of the component cells of the swordfish hypophysis. Those pars intermedia cells which stain with orange G are referred to as "pars intermedia acidophiles," etc.

The pars intermedia

The pars intermedia appears as a cone-like region with its apex directed ventrally which completely surrounds the arborized portion of the main nervous penetration. This region of the hypophysis is characterized by predominant weak basophiles which assume a dull blue color. These cells are small, being six to eight micra in diameter, and

are generally iso-diametric. Occasionally basophiles are found in the pars intermedia which are twice the usual size and possess small carminophilic granules distributed throughout the cytoplasm. These granules may be mitochondria, for those in proximity to the nucleus often assume rod-like forms. In addition to the basophiles, orange G acidophiles are also found in the pars intermedia, but these cells are much fewer in number. The cytoplasm of the acidophiles is of a dull orange color, with a fine granulation. Negative images of the Golgi apparatus are often seen in this type of cell. There is no definite arrangement of the acidophiles within the pars intermedia of the swordfish, as Scruggs (1939) and Bell (1937) found in the goldfish hypophysis, for they seem scattered at random throughout this part of the gland.

Degranulated cells, with an indistinct outline and a partially pycnotic nucleus, occur throughout the pars intermedia. These strongly resemble similar cells described by Scruggs (1939) for the pars intermedia of the majority of teleost pituitaries that he studied.

The Übergangsteil, or "transitional region"

The main portion of this part of the hypophysis is wedge-like in sagittal section, and lies dorsally to the base of the cone-shaped pars intermedia, with the main mass of tissue in the postero-dorsal corner of

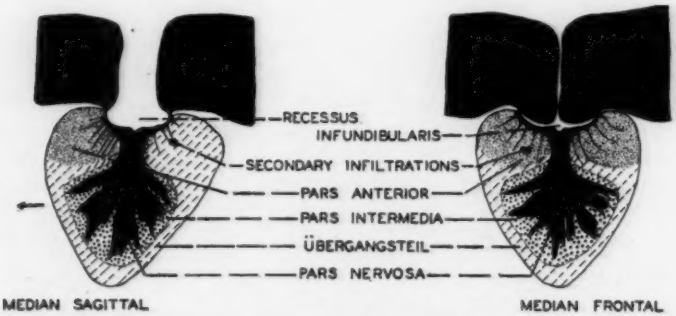

FIG. 1. Diagrams of median sagittal and median frontal sections of the swordfish pituitary gland, showing the topographical arrangement of the glandular regions.

the gland (Fig. 1). From this region paired processes extend anteriorly around the main nervous penetration to the frontal surface of the pituitary. A sheath of Übergangsteil material also descends over the pars intermedia, completely enveloping it and forming the superficial layer

of the hypophysis, excepting that portion of the gland where the pars anterior is situated.

The cells of the Übergangsteil are of two types; one is an acidophile and the other a basophile. The acidophiles are strong carminophiles, which react readily with and retain the azocarmine to assume a deep crimson color. Their cytoplasm is finely granulated, with larger granules dispersed through the cell. Usually the acidophiles are clustered about the capillaries, which are most numerous in this region of the gland; they also border the penetrations of infundibular material. The basophiles of the Übergangsteil, in those fish captured in midsummer, are of a brilliant blue color, with homogeneous cytoplasm and an exceptionally large cytoplasmic vacuole in each cell. (See plate.) The entire Übergangsteil appears net-like in sagittal section, due to the presence of these vacuolated basophiles.

The pars anterior

The pars anterior occupies a horse-shoe shaped region in the anterodorsal portion of the hypophysis, with its arms extending posteriorly around the zone of contact of the infundibulum with the pars glandularis to meet the main Übergangsteil mass. There are two types of cells, an amphiphile and an acidophile, in the pars anterior of the swordfish. The amphiphiles react polychromatically with the triple stain, showing a predominant tendency to assume the anilin blue which gives them a bluish-purple color. These cells line the infiltrations of infundibular material which penetrate this part of the gland. Their nuclei adopt a distal position with regard to the infundibular tissue; the intervening cytoplasm is very finely granulated and usually possesses small vacuoles.

The bulk of the pars anterior is composed of dark red carmine acidophiles. These are readily distinguished from the type of acidophiles in the Übergangsteil by means of their predominant color and coarsely granulated cytoplasm. In those acidophiles which approximate the numerous capillaries in the pars anterior, there is a definite concentration of these granules in the ends of the cells proximal to the blood vessels, with the nuclei occupying a distal position.

Histological Changes in the Pars Glandularis

During both summers that the swordfish pituitaries were studied, considerable changes were found in the aspect of the pars intermedia and the Übergangsteil, as the season progressed.

The pars intermedia of pituitaries obtained in early July possessed one to two orange G acidophiles per high power microscopic field (490 ×). The numbers of these cells gradually increased in pituitaries obtained successively throughout the summer, until there were 10 to 11 such acidophiles present per high power field in the pars intermedia of the last fish captured in early September. As the acidophiles increased in number, they appeared to be most numerous in the regions of the pars intermedia closest to the main branches of the pars nervosa which it surrounds. In this respect, the pars intermedia and its components in the swordfish agree more closely with the findings of Bell (1937) and Scruggs (1939) mentioned previously.

The basophiles of the Übergangsteil also showed marked changes in appearance during both summers. The aspect of these cells in pituitaries taken from late July to the end of the season has been described previously. However, there is a relatively complete absence of vacuoles in the Übergangsteil basophiles of pituitaries obtained in early July, and the cytoplasm of these cells possesses a coarse granulation. (See Plate.) As the sex of those swordfish from which these pituitaries were taken was undetermined, the exact nature and causes of the differences in aspect of the Übergangsteil basophiles still remain to be investigated.

Discussion

The topographical appearance of the swordfish hypophysis presents an interesting comparison with that of teleost pituitary glands that have been studied previously (Stendell, 1914; Bock, 1928; Charipper, 1936; Bell, 1937; Scruggs, 1939) with regard to the Übergangsteil. The generalized teleost pituitary is composed of two primary regions: that of nervous origin, the pars nervosa, and that of buccal origin, the pars glandularis. In the latter, the Übergangsteil develops as a plate or disc-like collar around the more dorsal portion of the pars nervosa, situated more or less completely between the pars intermedia and the pars anterior. However, in the swordfish the Übergangsteil is considerably flattened in its frontal portion, with the bulk of its tissue in the postero-dorsal region of the gland. A sheath of its components also invests completely the pars intermedia, to form the superficial layer of the hypophysis (Fig. 2). This condition suggests that in organogeny the process of intergrowth and differentiation of the pars glandularis with the pars nervosa is incomplete, as compared to the same process in other teleosts. Scruggs (1939) found a topographical condition similar to that of the swordfish hypophysis in the *Centrarchidae;* but in these fish the Über-

gangsteil sheath is fragmentary or completely lacking in the posteromedial line.

Because of differences in methodology, it is extremely difficult to compare the cellular components of the swordfish hypophysis with those of other teleosts that have been studied. A comparison of the nature of the subdivisions in the pars glandularis is probably valid, however, if made with regard to the predominant cell types present (basophiles, chromophobes, etc.), and to their distribution within the region in which they occur. Scruggs (1939) used the azan technique extensively on teleost pituitaries; hence the cellular components of the swordfish pitui-

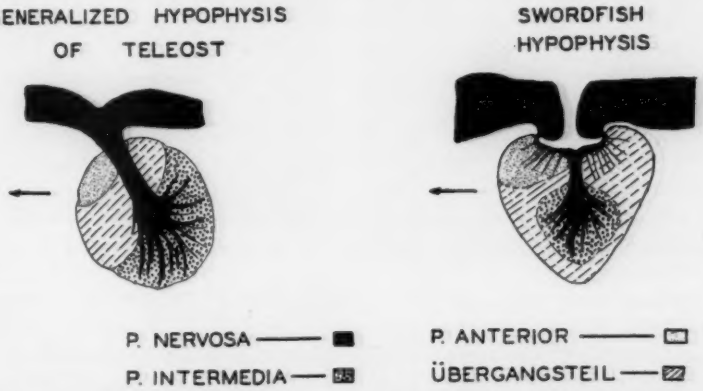

FIG. 2. Diagrams comparing the topographical arrangement of the glandular regions in the swordfish pituitary gland with that in the generalized teleost pituitary. (Anterior end to the left.)

tary gland may be compared more closely with those of the teleost glands which he studied.

The cell types of the pars intermedia compare favorably with those described by Charipper (1936), Bell (1937) and Scruggs (1939). Weak basophiles characterize the region, with weak acidophiles scattered through them, more or less at random. Occasional chromophobes are evidently formed by degranulation of the basophilic cells. The fact that Dawson (1940) found these three types of cells in the pars intermedia of *Protopterus aethiopicus* indicates that this region of the fish pituitary gland is relatively constant with regard to its cell components.

The components of the Übergangsteil also agree with the general condition found by other students of the teleost pituitary gland. Its brilliant carminophiles and deep blue basophiles are essentially the same

types of cells that Scruggs (1939) found in the Übergangsteil of all fish that he studied save the flounder and stickle-back. Charipper (1936), Kerr (1940) and others are agreed that this part of the teleost hypophysis is usually highly chromatic, possessing strong acidophiles and basophiles with the various staining techniques.

The predominantly chromatic nature of the pars anterior, however, is at variance with the condition that Scruggs (1939) describes for the large majority of fish that he examined. Bell (1937) has shown that the pars anterior of the goldfish is dominated by orange G acidophiles; while Kerr (1940) found this region of the trout pituitary to be chiefly acidophilic, possessing a brownish-red acidophile when stained with Mallory's triple stain. Thus the pars anterior of the teleost pituitary gland may vary considerably in different species and genera.

In its fibrous appearance the pars nervosa of the swordfish hypophysis resembles that of other fish that have been examined; however, the chromatic granules described by Scruggs (1939, 1942) for the pars nervosa of many species, and by Evans (1940) for the eel, are not found in the swordfish.

The changes in aspect of the Übergangsteil and pars intermedia of the swordfish hypophysis are without precedence in the literature. However, several investigators have reported various dissimilar phenomena in the pituitaries of other fish. Matthews (1939) and Scruggs (1939) have found several changes in the pituitary gland of *Fundulus heteroclitus* that are correlated with the beginning of the breeding season. Scruggs (1942) describes marked changes in the appearance of the pituitaries of *Cyprina carpio* and *Carassius auratus* that appear prior to and during the breeding periods of these fish. Evans (1940) reports large colloid-filled sinusoids in the pars intermedia of the eel, appearing

PLATE I

FIG. 3. The forward process of the Übergangsteil of a swordfish pituitary gland obtained on July 12, 1941. The darker cells are the bright crimson carmine acidophiles; the lighter cells are the granular basophiles. (720 ×.)

FIG. 4. The forward process of the Übergangsteil of a swordfish pituitary gland obtained on August 4, 1941. The basophiles possess large cytoplasmic vacuoles. Their nuclei are slightly pycnotic, and their cytoplasm has lost its granulation, to assume a dense homogeneous nature. This condition prevails for the remainder of the season. (800 ×.)

FIG. 5. The layer of amphiphiles in the pars anterior of a swordfish captured on July 24, 1940. The amphiphiles border an infiltration of connective tissue and blood vessels from the infundibular region. (1080 ×.)

FIG. 6. Carmine acidophiles bordering a blood vessel in the pars anterior of a swordfish captured on July 24, 1940. (The same fish whose pars anterior is shown in Figure 5.) The large carminophilic granules are concentrated in that end of the cells nearest to the blood vessel. (1080 ×.)

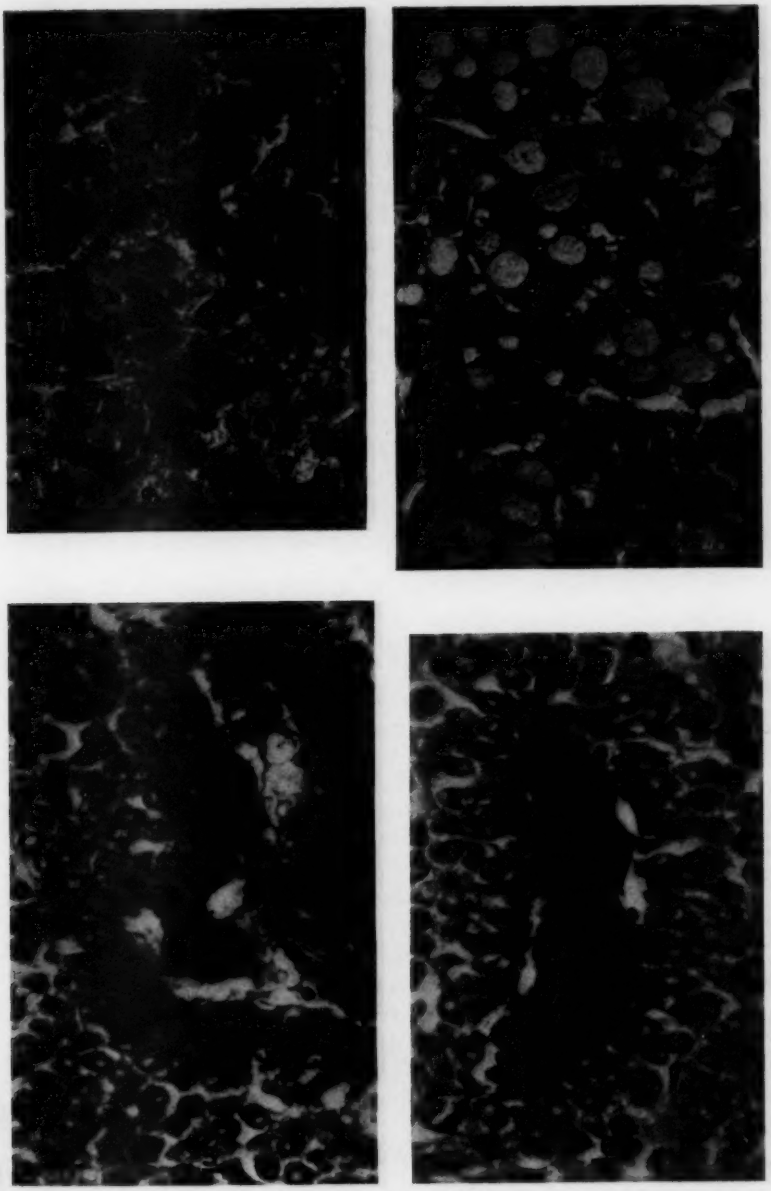

PLATE I

just before the onset of the migration impulse, with an enlargement of the pars anterior found at approximately the same time. Although these changes are not similar to those found in the pituitary of the swordfish, they indicate the components of the teleost pituitary gland may undergo a considerable modification which is apparently correlated with the breeding period. As the ovaries of the swordfish show no indication of maturation or of an increased pituitary activity during the brief time that the fish is found in southern New England waters, and as the exact nature of the changes found in its pituitary gland is still to be established, we can draw no conclusions regarding the time or the regions of breeding activity of the swordfish at present. Future examinations of the reproductive condition of this fish in all portions of its range will undoubtedly aid in the solution of this problem.

I am deeply in debt to Dr. A. B. Dawson of the Harvard Biological Laboratory for encouragement and aid in pursuing this study; to the staff members of the Woods Hole Oceanographic Institute, for their generosity in providing laboratory space and equipment; and to Mr. Samuel Cahoon of Woods Hole, whose facilities and knowledge of swordfishing were invaluable in securing the material.

Summary

Twenty-three swordfish pituitaries were studied in 1940. It was impossible to determine the sex of these fish; therefore the work was repeated in 1941 on pituitaries from 13 swordfish, all of which proved to be female. Their pituitaries were identical with those obtained in 1940.

The swordfish hypophysis is a blunt pyramidal structure, situated with its base in close approximation with the infundibulum. The pars nervosa descends from the infundibular floor, as a main or primary infiltration which arborizes in the ventral portion of the gland, with smaller secondary infiltrations of infundibular tissue penetrating the more dorsal regions. It is fibrous in nature, with the nuclei of connective tissue cells occurring frequently. Herring's bodies are not found in the pars nervosa, and there is no evidence of migration of cells from other glandular regions into it.

The pars intermedia surrounds the arborized part of the main nervous penetration, as a cone-shaped region with its apex directed ventrally. There are three types of cells in this part of the gland; an orange G acidophile, a weak blue basophile, and a chromophobe. Carmine granules are found in many of the basophiles. The acidophiles

become more numerous in glands obtained successively during the summer.

The Übergangsteil is a wedge-shaped region, lying dorsal to the base of the cone-like pars intermedia, with its main portion in the postero-dorsal part of the hypophysis. A sheath of its material completely envelops the pars intermedia. The Übergangsteil possesses two types of cells: a strong carminophile with finely granulated cytoplasm, and a deep blue basophile with coarsely granulated cytoplasm. During the latter half of the summer the basophiles contain large vacuoles which first appear about the middle of July.

The pars anterior occupies a horse-shoe shaped region in the antero-dorsal part of the hypophysis. Its arms extend posteriorly toward the main mass of the Übergangsteil, often surrounding the zone of contact of the infundibulum and the pars glandularis in collar-like fashion. The pars anterior of the swordfish pituitary has two chief types of cells: an amphiphile and an acidophile. The former are of a bluish-purple color, and are found bordering the secondary infiltrations of infundibular material that enter this region. The acidophiles are coarsely granulated carminophiles, which lie beneath the more peripheral amphiphiles to make up the bulk of the pars anterior.

The 13 swordfish from which gonads were obtained in 1941 were females. The ovaries contained ova which did not change appreciably in diameter during the summer. Thus there is no evidence of sexual maturation of swordfish taken in southern New England waters.

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ACCUMULATION AND DISCHARGE OF SPAWN BY OYSTERS LIVING AT DIFFERENT DEPTHS

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Introduction

While conducting studies on the biology of the oyster, *Ostrea virginica*, of Long Island Sound, an opportunity presented itself to observe and compare the condition of the gonads of these animals living at different depths. Such a study is of interest since the literature does not offer any comparative data pertaining to the accumulation of spawn and spawning activities of oysters existing in shallow and deep water. There are in the literature, however, several references which indicate that the oysters of shallow water discharge their spawn earlier than those of deep water. For example, Churchill (1920) states that the spawning period of *O. virginica* is regulated by the depth of the water and the general meteorological conditions. Nelson (1928), working in New Jersey, also expressed the opinion that oysters in shallow water spawn before those in greater depth. Neither of the two above-mentioned investigators, nor other workers making similar statements, offered any comparative data in support of their observations. Furthermore, no suggestion was offered as to what should be considered as shallow or deep water.

In Long Island Sound very shallow waters are not suitable for oyster cultivation because wave action during storms may shift and often destroy the oysters. On the other hand it becomes increasingly difficult to cultivate the beds and take care of the oysters in waters deeper than 30 or 35 feet. As a result of these conditions the greater part of the oyster grounds of Long Island Sound is confined to the depths ranging from 10 to 30 feet. Therefore, in these studies it was decided to confine observations to the same depth range.

Methods

These studies were conducted in the summer of 1941. There were 14 sampling stations established in the oyster-growing area confined between Bridgeport and New Haven harbors. Of this number, four stations were located at a 10-foot depth, six stations at a 20-foot depth,

and four stations at a 30-foot depth. It is proposed to regard the above mentioned groups of stations as shallow, medium deep, and deep, respectively.

The collection of samples for observations on the average thickness of the gonadal layer of oysters living at the three different depths was begun on June 11 and continued until September 3. At each station a dredge-load of oysters was brought up and a sample of ten adult animals of four or five years of age was taken from it at random. Thus, 140 individuals were examined on each trip. The body of each oyster was cut transversely on the right side along a line extending through the stomach on a level with the lower edge of the palps, and the thickness of the gonadal layer was measured with a caliper. To determine the time of the beginning of spawning of the oyster population, numerous other samples were taken at very frequent intervals during the early part of July.

Several days after the beginning of spawning, collection of additional samples of oysters for determination of the condition of their gonads was initiated. Each sample now consisted of 20 individuals: thus during each trip 280 oysters were collected from 14 stations. The oysters were opened, dissected, and the condition of their gonads determined. On the basis of such an examination the oysters were classified as ripe-but-unspawned, less-than-half spawned, more-than-half spawned, and completely spawned.

Observations

The first measurements of the gonads of oysters made on June 11 showed that the gonadal layer was very thin and that the majority of the animals was unripe. Oysters of the shallow stations were the most advanced, the mean thickness of the gonadal layer of that group being slightly over 1.0 mm. (Fig. 1). Two other groups of oysters collected from the stations located at depths of 20 and 30 feet showed the thickness of the gonadal layer as about 0.9 and 0.8 mm. respectively. In all groups, especially in that of the deep water stations, there were individuals whose gonads only recently had changed from the wintering stage, and whose gonadal layer was extremely thin. Later in June the increase in the thickness of the gonadal layer of all three groups of oysters became quite rapid, and early in July, before the beginning of spawning, the gonads reached their maximum development.

Throughout the prespawning period the oysters of the more shallow stations possessed a heavier layer of spawn than the oysters from deeper water (Fig. 1). This difference became more apparent as the season

progressed toward the commencement of spawning. On July 2 the thickness of the gonadal layer of shallow water oysters was approximately twice that of oysters from the 30-foot depth. Oysters of medium deep stations occupied a position between the extremes.

Because the last measurement of gonads during the prespawning period was made on July 2, approximately one week before the beginning of spawning, it must be assumed that during that last week a further increase in thickness of the gonadal layer occurred. Therefore, it is probable that the maximum thickness of the gonadal layer of the oysters at all three depths was greater than is shown in Figure 1. However, judging by the examination of unspawned individuals collected on July

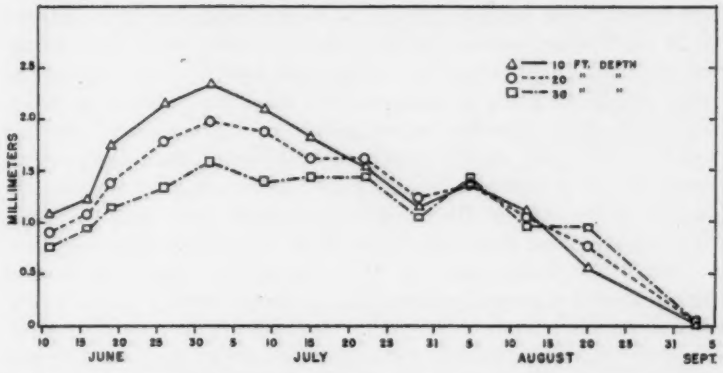

FIG. 1. Mean thickness of the gonadal layer of the oyster at three different depths.

9, the increase in thickness of the gonadal layer during the week elapsing between July 2 and 9 was very small.

In 1941, the beginning of spawning of the oyster population occurred about July 9. Spawning was of a general nature taking place at all the stations regardless of depth. After the beginning of spawning the thickness of the gonadal layers of oysters at all the depths began to decrease. While such a decrease was rather slow and gradual, it was most pronounced in the case of the shallow water oysters (Fig. 1). This, of course, indicated that the shallow water group discharged the spawn in larger quantities and more rapidly than the oysters of deeper water. As a result of the uneven rate of the discharge of spawn, the thickness of the gonadal layers of the oysters of all three depths became actually the same by July 22. Later on, the shallow water oysters began to possess less spawn than the two other groups (Fig. 1).

On July 15, several days after the beginning of spawning, a series of observations on the condition of gonads of the oysters was begun. It was found that the majority of the oysters at all the stations had already released part of their sex cells. It was of interest to note that, contrary to expectations, there were fewer ripe-but-unspawned oysters at the deep water stations than at more shallow ones (Fig. 2*A*). In all cases, nevertheless, the majority of oysters examined at this date were in the less-than-half spawned stage. Such animals constituted 71.0, 61.7 and 81.3 per cent of the samples collected at depths of 10, 20 and 30 feet respectively (Fig. 2*B*). More-than-half spawned oysters were most numerous at the 20-foot depth where they constituted 18.3 per cent of the total number of oysters examined (Fig. 3*C*). Completely spawned oysters were absent in the samples collected at medium-deep and deep stations, but at the shallow stations 1.6 per cent of oysters were identified as entirely spent (Fig. 3*D*). This observation indicated that among the oyster population living in shallow water there was a small group of individuals whose spawning season lasted but a single week. Several oysters of this type were prepared for histological examination which showed by the shrinkage of the gonadal follicles, phagocytosis, and the invasion of the cells of the vesicular connective tissue into the intra-follicular spaces that their gonads were in the typical post-spawning stage.

Within two weeks after the first examination, ripe-but-unspawned oysters showed a marked decrease in numbers. Such a decrease was noted at all three depths (Fig. 2*A*). Nevertheless, unspawned individuals were found in the samples collected at the 10-foot depth until August 12, and at the two greater depths until August 20. However, between July 29 and August 20, ripe-but-unspawned oysters never constituted more than 2.6 per cent of the population at any depth.

Early in August, approximately 50 per cent of the oyster population had half its spawn already discharged (Fig. 4). Again it was noted that the shallow water oysters, living at the 10-foot depth, reached this stage several days earlier than the animals living in deeper water (Fig. 2*B*).

As is shown in Figure 2*B*, the half-spawned stage was reached by the oyster population sometime between August 5 and 12. To determine a more exact date for the midpoint of the spawning period, the following method was employed: the oysters composing ripe-but-unspawned and less-than-half spawned classes were combined in one group, while two other classes, in a more advanced condition, were taken as the second group. By employing such a classification all oysters were divided into two groups of which the first contained all less-than-half spawned

oysters, while the second group was composed of the individuals which had more than half their spawn already discharged. The last group, of course, included all completely spawned oysters. When the percentage of these two groups was plotted against the time, two curves intersected at the point corresponding to August 8 (Fig. 4). Therefore, it can be assumed that this date represents the midpoint of the spawning season or, in other words, the date at which the oyster population of the Sound had discharged approximately one-half its spawn.

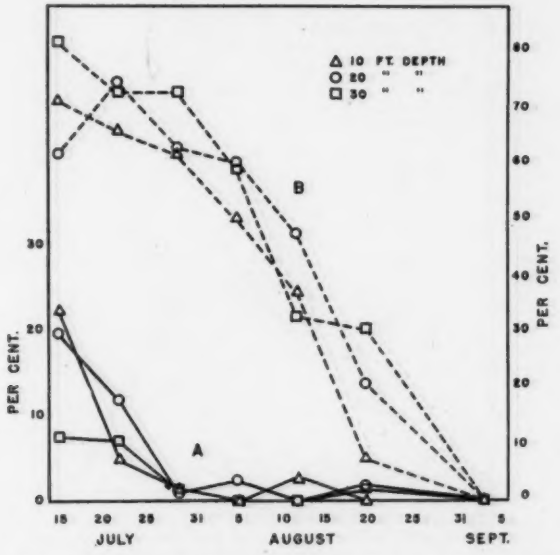

FIG. 2. Percentage of ripe-but-unspawned (*A*), and less-than-half spawned (*B*) oysters at each of three depths.

The conclusion that the midpoint of the spawning season lies close to the date of August 8 is further supported by the data obtained from the measurements of the thickness of the gonadal layer of oysters (Fig. 1). These measurements showed that the average thickness of the gonadal layer of the oysters recorded several days before the beginning of spawning was about 2.0 mm. On the other hand, the average thickness of this layer, as determined on August 12, the date nearest to August 8, was only about 1.0 mm., or approximately one-half that at the time of the maximum development.

Individual oysters containing more than half the spawn were still quite numerous in the samples long after the midpoint of the spawning period was reached (Fig. 2*B*). Even as late as September 20, 32.5 per cent of the oysters living at the depth of 30 feet was found in this stage. At more shallow stations their numbers were smaller.

As was stated before, few completely spawned oysters were found in the samples as early as July 15. Those animals, however, came from

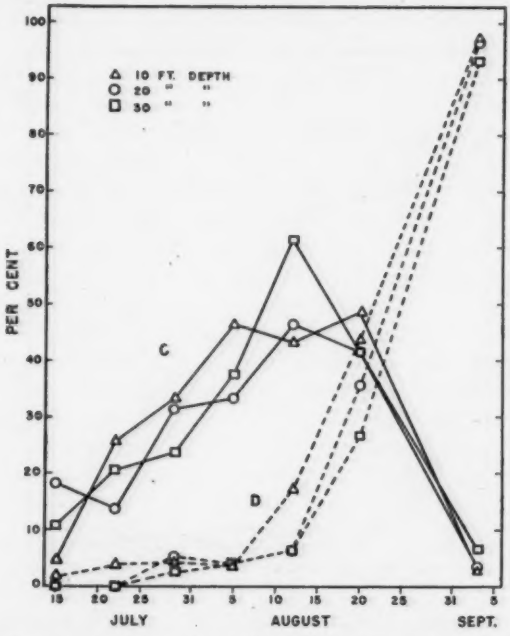

FIG. 3. Percentage of more-than-half spawned (*C*), and completely spawned (*D*) oysters at each of three depths.

shallow water. At deeper stations located at the 20- and 30-foot depths, completely spawned animals were first found on July 29 or two weeks later than at the shallow stations. At that date their numbers were comparatively small and never constituted more than 5.1 per cent of the entire population (Fig. 3*D*). A significant increase in the numbers of completely spawned oysters of shallow water was noted by August 12, when they constituted 17.1 per cent. At the two other depths, large numbers of spent oysters were observed one week later, when they constituted 35.8 and 26.6 per cent of the population living at 20- and 30-foot

depths respectively. After August 20, the number of completely spawned oysters rapidly increased and on September 3, at the end of the observation period, over 95.6 per cent of the entire population belonged to that group (Figs. 3 and 4). The remaining 4.4 per cent represented the animals still containing small quantities of spawn and, therefore, considered as more-than-half spawned but not entirely spent. It may be

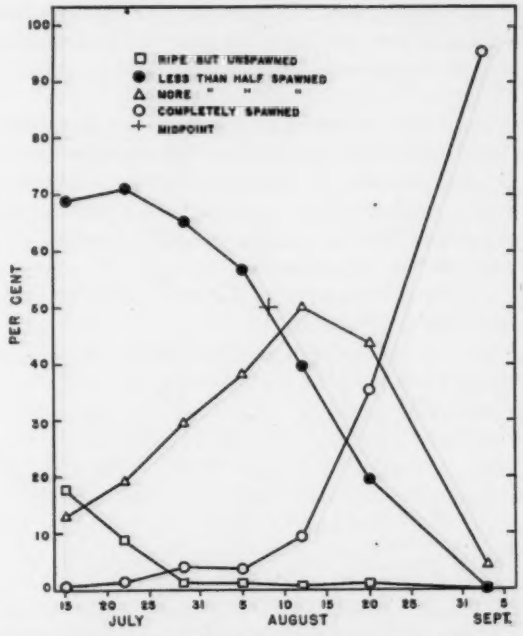

FIG. 4. Percentages of ripe-but-unspawned, less-than-half spawned, more-than-half spawned, and completely spawned individuals composing the oyster population of the depths ranging from 10 to 30 feet. July 15 to September 3, 1941. Cross sign (+) indicates the midpoint of the spawning season.

concluded, however, that the spawning activities of the majority of the oyster population living at the depths ranging from 10 to 30 feet were finished about September 3.

DISCUSSION

The observations conducted in 1941 in Long Island Sound indicated that, within the depth range of from 10 to 30 feet, the spawning of the oyster population began at the same time. This observation is in agree-

ment with those of previous years when it was found that the oysters of shallow and deep water beds approached ripe conditions and discharged spawn on or about the same date (Loosanoff, 1939; Loosanoff and Engle, 1940). The spawning season continued from about July 9 until about September 3. The shallow water oysters finished spawning somewhat earlier than the oysters living in deeper water.

It was determined that the midpoint of the season, or the date by which the oyster population had discharged approximately one-half the spawn accumulated, was reached by August 8. Again shallow water oysters reached this point several days earlier than the animals living in deeper water.

It is of interest to know that, among the oyster population studied, there was a small group found whose spawning activities were begun and ended within a week's time. It is possible that these individuals represent a race physiologically different from other local oysters whose spawning periods extend about two months. It has been suggested that such individuals are the descendants of the oysters brought on several occasions to Long Island Sound from northern waters where the spawning season is of very brief duration.

The presence of ripe-but-unspawned oysters, encountered in the samples collected six weeks after the beginning of the spawning season, presents an interesting problem. The failure of some individuals to spawn until very late in the season was first noticed during the spawning season of 1937 (Loosanoff and Engle, 1940). Physiological ripeness of such a late-spawning type of female oysters was established by fertilizing their eggs with spermatozoa taken from the males of the same late-spawning group. Therefore, it appears that under natural conditions some of the ripe oysters cannot be induced to spawn by a relatively high temperature and by the chemical stimulation caused by the presence in the water of the sex products discharged by the oysters living nearby. These field observations are supported by the laboratory experiments of Galtsoff (1940) who found that from 5.4 to 22.8 per cent of males and from 4.7 to 29.4 per cent of females could not be induced to spawn by the combination of thermal and chemical stimuli. Histological examination of these animals showed that a great majority of them was ripe.

The observation that the oysters living in shallow water develop much larger quantities of spawn than the oysters of greater depths is of considerable significance. It indicates that during the prespawning season it may be advantageous to have large numbers of oysters concentrated in relatively shallow water. In that case, theoretically, each oyster would produce about twice as many sex cells as it would if it were kept

at the depth of 30 feet. At present no satisfactory explanation can be offered as to why the shallow water oysters accumulate larger quantities of spawn. At first the difference in water temperature was suspected as causing much more favorable conditions of existence for shallow water individuals. Field records showed that during the prespawning period, the bottom water temperature at shallow stations was from only 1° to 2° C. higher than at the 30-foot depth. Therefore, even if the difference was actually insignificant, the assumption could be made that the shallow water oysters accumulated more spawn because they lived in warmer water. Such an assumption, however, would be contradictory to the observations of the last nine years which showed rather conclusively that the quantity of spawn developed by oysters in different years could not be correlated with the deviations of temperature during spring and early summer above or below normal (Loosanoff and Engle, 1940). Furthermore, minor differences in temperature have been found to be unimportant in other physiological activities of oysters. For example, Galtsoff (1928), in well controlled experiments performed on a large number of oysters, found that within temperature limits ranging from 13° to 22° C. there was no visible effect of temperature on the opening and closing of shells. Recent experiments on the feeding of oysters conducted by the authors at Milford Laboratory failed to show that, within the temperature range mentioned above, there was a well defined decrease or increase in the rate of feeding of oysters when the temperature of the water was changed by one or two degrees.

The differences in the salinity of the water at the stations of the three different depths were rather small seldom exceeding one part per mille. Evidently such a difference was not significant enough to cause much heavier development of the gonad material in the oysters living at one of the depths. It is thought, therefore, that some other conditions are responsible for the accumulation of greater quantities of spawn by shallow water oysters. The suggestion is offered that it may be due to the presence of larger numbers of food organisms in the shallow zones. Unfortunately the present knowledge of the feeding habits of oysters is rather limited. Even though there are many articles describing the plankton organisms found in the oyster's stomach, little is being said in these articles on the digestion and assimilation of such forms. As a result, very little information is available as to the quantities and qualities of various food organisms assimilated by oysters. Therefore, this phase of the biology of the oyster is not clearly understood. Obviously, in order to ascribe the greater gonad development of oysters of certain depths to their feeding habits and the presence of certain food organisms, further research along these lines is necessary. At present a number of

experiments and observations devised to provide the possible answer to such problems are being conducted at the Milford Laboratory.

Summary

1. Prior to spawning the thickness of the gonadal layer of shallow water oysters was approximately twice that of the oysters living at a 30-foot depth.

2. The spawning of oysters at all depths began on and about July 9.

3. The shallow water oysters discharged spawn in larger quantities and more rapidly than the oysters of deeper water.

4. Among the oyster population living in shallow water there was a small group of individuals whose spawning was completed within a week.

5. Ripe-but-unspawned oysters were found in the samples collected as late as six weeks after the beginning of the spawning season. This indicates that some of the ripe oysters, living under natural conditions, cannot always be induced to spawn by a relatively high temperature and or by the chemical stimulation caused by sex products discharged by the oysters living in the immediate vicinity.

6. Approximately 50 per cent of the oyster population had half its spawn discharged by August 8. This date, therefore, is considered as the midpoint of the spawning period. Shallow water oysters reached the half-spawned stage several days earlier than the deep water individuals.

7. The spawning activities of the oyster population, living at depths ranging from 10 to 30 feet, were finished about September 3. The majority of the shallow water oysters completed their spawning somewhat earlier than those of deep water.

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THE NORMAL GROWTH AND RESPIRATION OF TETRAHYMENA GELEII

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The early population growth phases of *Tetrahymena geleii* (Furgason, 1940) are well established due to the work of Elliott (1933), Phelps (1935), and Hetherington (1936). The later growth phases are relatively unexplored. Phelps (1935, 1936) outlined the effect of food concentration and of the age of the inoculum. He found that Buchanan's (1918) lag phase, exponential phase, decreasing acceleration phase, and stationary phase were present in the growth of bacteria-free cultures of this ciliate and unlike most bacterial populations the stationary and lag periods were not influenced by the size of the inoculum. Varying the size of the inoculum varied only the length of the exponential phase. He demonstrated that within wide limits the concentration of food had no effect on the rate of growth in the exponential phase but only on the maximum concentration.

No complete explanation for the appearance of these various population growth phases is yet available. Food certainly plays a part, although by no means is it the only agent. Since Phelps found that in yeast autolysate concentrations between 0.4 per cent and 20 per cent there is a direct proportion between the food concentration and the maximum yield of ciliates, any factor such as Bail's (1929) "population pressure" or the presence of toxic excretory products would seem to be ruled out. Monod (1935) was likewise unable to demonstrate the appearance of any effects due to toxic substances. Kidder (1941b) suggests that an inhibitory substance as well as an accelerative factor may be produced as a result of a series of experiments on growth in "biologically conditioned" media. The oxygen tension has an effect on the length of the exponential phase and also on the maximum yield (Phelps, 1935) while the carbon dioxide tension apparently is not a limiting factor in cultures of *Tetrahymena geleii* (Jahn, 1936). The pH of the medium can influence the growth as shown indirectly by the respiration

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measurements of R. H. Hall (1941) and directly by numerous authors including Elliott (1933), Johnson (1935) and Kidder (1941a).

There has been no thorough examination of the relationships existing between population growth and cell growth in the various growth phases of protozoan cultures. These relationships become important in respiration and metabolic studies in which the information needed is the activity per unit of protoplasm rather than per organism. A study has been made therefore of the relationships existing between cell growth and population growth. This data has been correlated with normal oxygen consumption in the various population-growth phases.

It will be shown that changes in cell size may occur during the lag and early exponential population-growth phases depending on the conditions of the experiment and that consequently there may be an increase in total protoplasm due to cell growth rather than combined cell and population growth. It will likewise be shown that characteristic cell-size changes can be correlated with other population growth phases. The behavior of populations in very old cultures will be briefly noted.

This work was done at the Arnold Biological Laboratory, Brown University, and the Marine Biological Laboratory, Woods Hole, Massachusetts. I wish to express my sincere appreciation to Dr. George W. Kidder for his helpful suggestions and sustaining interest.

Material and Methods

The organism used in these experiments was *Tetrahymena geleii* (Hetherington strain). In the experiments on the early growth phases cultures were grown in 250 ml. Kidder culture flasks (Kidder, 1941b) in 100 ml. lots of 2 per cent Difco proteose peptone made up with Pyrex-distilled water. Cultures used in the work on size and population changes over long periods of time were grown in liter flasks using 500 ml. of culture fluid.

All cultures were grown in an incubator at 26° ± 0.3° C. and under sterile conditions. Sterility was checked by inoculation onto nutrient agar slants and Petri dishes which were incubated at 26° ± 0.3° C. for two weeks before a final diagnosis of sterility was made. All contaminated cultures were discarded. In the work on population and size changes over long periods the cultures were checked after each withdrawal of a sample, and at the end before the flasks were discarded.

Population counts were made by direct counting of the live organisms following serial dilution in 2 per cent proteose peptone until the concentration of the ciliates was between 25 and 125 organisms per 0.2 ml. This amount was then streaked out on glass slides and counted with a

binocular dissecting microscope. The error involved in this method amounts to ± 5 per cent.

In the work on size changes the organisms were killed by fixation in 5 per cent formalin and measured with an ocular micrometer. No appreciable shrinkage due to the formalin was observed.

Dry weight measurements were made by centrifuging the suspension of organisms, removing the supernatant and washing three times with distilled water. Ten ml. of the final suspension were then pipetted into a porcelain crucible which was placed in an oven at 100° ± 0.5° C. for 24 hours. They were then removed to a desiccator until the weights became constant.

Nitrogen determinations of washed organisms were made by the micro-Kjehldahl method. The apparatus used is described by Pregl in Roth's (1937) translation. The method used was that of Bang (1916), Parnas and Wagner (1921) as modified by Elek and Sobotka (1926).

Respiration measurements were made with the standard Barcroft-Warburg apparatus. The technique employed is outlined in Dixon (1934). Vessels of about 10 ml. capacity were used with a total of 2 ml. of fluid in each. Carbon dioxide absorption was by means of a roll of Whatman (starch-free) No. 40 filter paper soaked with 0.2 ml. of 20 per cent KOH. The apparatus was set to give 140 oscillations per minute with a stroke of 4 cm. Temperature in all determinations was maintained at 26.8° ± 0.1° C.

The suspensions of organisms used in respiration determinations were made up of organisms resuspended in a 0.005 M phosphate buffer of pH 6.9. The pH was checked at the end of each experiment. The density of the suspensions used was between 100,000 and 500,000 ciliates per ml. Within these limits neither the rate of shaking nor the amount of potassium hydroxide was a limiting factor in the determinations of oxygen consumption. Oxygen consumption is expressed as cubic millimeters of dry gas under standard conditions of temperature and pressure.

Experimental Results

Normal Growth

The normal population growth curves obtained were similar to those obtained by Phelps (1935, 1936) and Kidder (1941a). Cultures were set up using 2 per cent proteose peptone in Kidder culture flasks as previously described. A normal curve (*A*) is shown in Figure 1. The lag period is not shown. The interdivisional period during exponential

growth was 3.32 hours for this set of experiments. Population counts were obtained by using as the diluent conditioned 2 per cent proteose peptone in which ciliates had been grown for the same length of time as those in the experimental flask. The organisms were killed by gentle heating in a flame, centrifuged out, and the supernatant used as the diluent. Following dilution of a sample of the experimental culture the dilutions were allowed to stand for ten minutes following which the counts were made. When a fresh 2 per cent proteose peptone was used as the

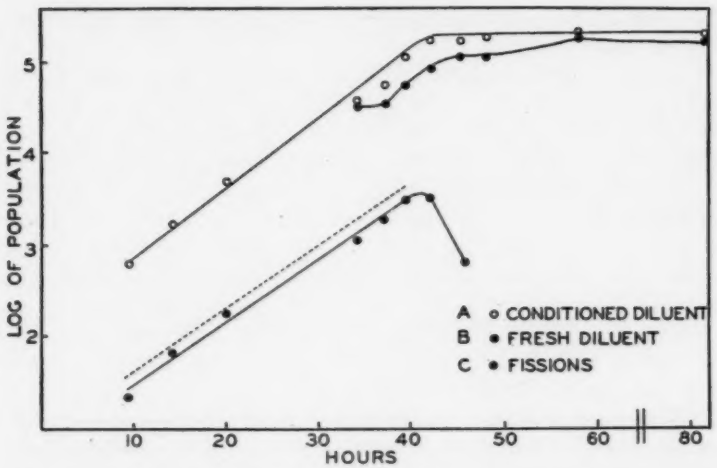

FIG. 1. Population and fission curves in 2 per cent proteose peptone. The dotted line represents the theoretical fission curve. All curves are on the same ordinate.

diluent, curve *B* was the result. This curve indicates the presence of an increased sensitivity of the organisms as the population approaches the end of the phase of exponential growth. This sensitivity was not apparent in the early exponential phase and gradually disappeared in the hours following the end of the exponential phase. It was later found that accurate counts (5 per cent) could be made using fresh proteose peptone as the diluent if the diluting and counting procedure were completed within five minutes.

The average fission time will of course vary with the criteria of the investigator. In this study fission was arbitrarily assumed to have begun when the first external indication of the cytokinesis could be observed using a dissecting binocular microscope with 10 × oculars and

10 × objectives. The average of 20 determinations of fission time at room temperature and in hanging drop preparations was 12.5 minutes. Curve *C* in Figure 1 represents the logarithm of the number of fissions in the exponential phase and in the hours immediately following it. The dotted line in Figure 1 represents the theoretical fission curve during the exponential phase.[2] As would be expected, the rate of increase of fissions is constant. The results are summarized in Table 1.

TABLE 1

Fission rate compared with the rate of population growth. Eight experiments. The theoretical number of fissions during the first 40 hours is 0.044 per cent.

Time in hrs.	Population/ml.	Fissions/ml. experimental	Per cent fissions experimental
10	594	22	0.038
14	1,585	63	0.040
20	4,740	168	0.038
34	30,600	1,060	0.035
37	53,100	1,780	0.034
40	89,100	2,982	0.034
43	141,300	3,160	0.022
46	149,700	595	0.004
49	173,800	—	—
59	211,400	—	—
82	210,000	—	—

It will be noted from Figure 1 that the percentage of fissions drops very rapidly when exponential growth ceases. After 46 hours the percentage of fissions was only 0.004. Beyond this point the number was so low that examination of 40,000 to 50,000 ciliates would not show enough fissions to form an accurate percentage figure. This indicates that in the stationary phase there is negligible death occurring. The growth rate of practically zero does not represent a balance between an increase due to fission and a decrease due to death of some organisms but an actually stationary population.

This conclusion is reinforced by a consideration of Figure 2, the data for which are contained in Table 2. Each length given in Table 2 repre-

[2] The population growth during the exponential phase may be described by the equation (1) $N = N_0 e^{\lambda G}$ where N = the number of individuals per unit volume, $\lambda = \log_e 2$ and G = the interdivisional period. The number of organisms at any time in the exponential phase *exclusive* of those in fission may be described by (2) $N' = N_0 e^{\lambda(G-S)}$ where S = the average time occupied in fission by each organism. Subtracting (2) from (1), and rearranging in terms of G, the percentage of individuals in fission at any time during the exponential phase is (3) $\frac{2^{S/G} - 1}{2^{S/G}}$.

TABLE 2

Cell growth compared with population growth. Size data from 14 experiments; each point represents average of 150 measurements. Weight determinations each represent average of three or more determinations. The MC signifies the end of the period of exponential growth.

Time in hrs.	Population/ml.	Length in micra	Time in hrs.	Wt. in mgm. per 10^6 organisms
0	100	74	10	5.32
4	120	68	MC	—
11	1,000	58	10	5.85
16	2,510	53	20	7.34
26	31,600	53	30	8.01
MC	—	—	40	—
3	123,200	57	50	8.34
19	135,000	70	60	8.79
26	158,600	73	70	8.82
40	162,500	75	80	—
75	174,300	75	90	8.85
100	128,800	75	130	8.55[a]
130	120,000	75	191	9.04[b]
145	102,700	71	—	—
169	100,100	70	—	—
191	89,200	69	—	—

[a] One determination. [b] Two determinations.

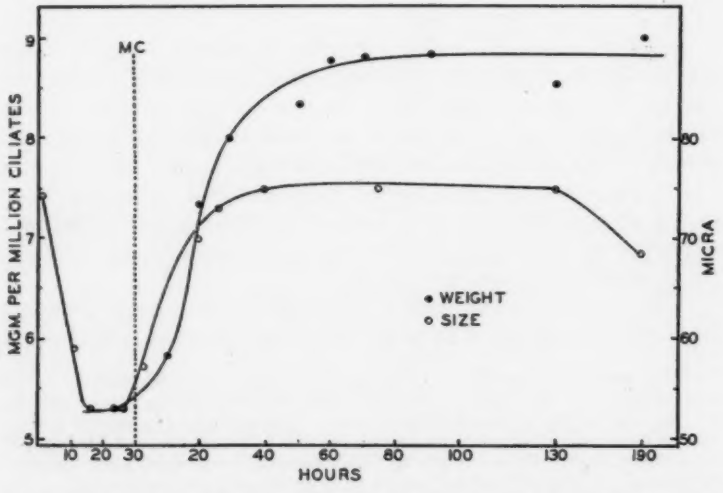

FIG. 2. Size and weight changes in 2 per cent proteose peptone flask cultures of *Tetrahymena geleii*. The abscissa represents the age of the culture.

sents an average of 150 measurements. The length was taken as an adequate criterion of the size, since the ratio of length to width did not vary significantly over the time range examined. The weight measurements are the average of 36 determinations and are plotted as the average of 10-hour intervals. Data for both weight and size are translated into terms of a 30-hour exponential period for purposes of comparison, and all determinations made after the exponential phase were calculated with the end of that period as the point of departure. Data such as these are significant in terms of the age of the culture with respect to the growth

TABLE 3

Nitrogen determinations. Three determinations were made on urethane-treated organisms in a separate experiment not reported here.

Dry wt. in mgm. of organisms	N in mgm.	Per cent N	Age of organisms in terms of 30 hour MC
27.1	3.1	11.4	23 hours
67.5	4.8	7.1	84 hours
62.6	6.5	10.4	93 hours
64.6	7.3	11.3	110 hours
22.1	2.2	10.0	127 hours
27.3	2.9	10.6	40 hours
12.9	1.3	10.8	47 hours[a]
14.0	1.6	11.4	34 hours[b]
71.7	7.7	10.7	87 hours
12.4	1.4	11.3	59 hours[c]
	Average	10.5	

[a] 13 hrs. in 1 per cent urethane.
[b] 11 hrs. in 1 per cent urethane.
[c] 25 hrs. in 1 per cent urethane.

phases and not in terms of absolute time units. With different-sized inocula the length of the exponential phase in these experiments varied from 15 to 45 hours. All weight measurements made on exponential phase organisms, when translated into terms of a 30-hour exponential growth period, came at the same place in time, e.g., 25 hours. Since they all agreed very closely it is clear that the weight of the individual ciliates is quite constant during the exponential phase. The time relations in the increase in size and weight following the end of the period of exponential population growth were comparable, regardless of the length of the exponential period. This demonstrates that size and weight measurements on organisms such as these must be judged with relation to the normal population curve and not by an absolute time scale.

Nitrogen determinations (Table 3) indicate a constant nitrogen per-

centage of 10.5 per cent. In other words, the increase in weight shown in Figure 2 represents an increase in protoplasm and not merely an increase in stored carbohydrate or something of like nature. The average nitrogen content agrees well with the scanty data available. Panzer (1911), in an analysis of the coccidian *Gaussia gadi*, found the nitrogen to be 9.7 per cent of the dry weight. Hutchens (1941a) reports the nitrogen content of *Chilomonas paramecium* to be 10 to 11 per cent.

In the lag and early exponential phases the shape of the size curve in Figure 2 depends upon the size of the ciliates used for inoculation of the culture. If organisms 50 to 75 hours old are used they are usually about 75 μ long. Upon inoculation into fresh media and the onset of division, the average size progressively decreases until a constant size of around 53 μ is reached. This size is then maintained to the end of the exponential period. On the other hand if organisms from a 30- to 40-day old culture (about 35 μ long) are used for the inoculation, there is an *increase* in average size until the constant size of about 53 μ is reached. Thus it is clear that in some cases at least the changes in individual size preceding and during cell division must be interpreted in relation to the age of the organism.

Population changes were followed for periods up to 90 days and cell-size changes up to 45 days in 2 per cent proteose peptone cultures. In cultures in which the *MC* occurred at 36 hours, the stationary phase of population growth persisted for five days, following which a phase of declining population set in. This phase was sometimes marked by rather sudden increases in population although the overall picture was one of gradually decreasing population. It is clear that the latter parts of Buchanan's classical population curve are not present in cultures of this type and in the time range examined. There is no clear phase of accelerated death or exponential death and the population decrease that is observed is not necessarily an even decline.

Cell size in cultures of this type, after increasing to a maximum of 75 μ in length as shown in Figure 2, maintained the maximum size for four days after which there was a gradual decrease. In three experiments this decrease in size continued for 34 days at which point there was no further change for six days when the experiments were discontinued. The ciliates had decreased to 36 μ length in these 40-day old cultures.

Normal Respiration Rates

Table 4 gives the results of normal respiration measurements on ciliates in 0.005 M phosphate buffer and in a 2 per cent proteose pep-

tone medium buffered with 0.005 M phosphate. The Q_{O_2} is defined as cu. mm. O_2/hr./mgm. dry weight of tissue. The exponential phase ciliates were taken between 20 hours and 30 hours from cultures in which exponential growth would have ceased at 30 hours. The stationary phase organisms were taken at around 50 hours from similar cultures.

Provided the concentration of organisms in the suspension measured is above the maximum concentration obtained in a normal culture, the

TABLE 4

Normal respiration values. The figures in parentheses refer to the number of determinations made. The Q_{O_2} represents cu. mm. O_2/hr./mgm. dry weight of ciliates.

	Exponential phase organisms		Stationary phase organisms	
	In 0.005M PO_4 buffer (65)	In 2% proteose peptone (11)	In 0.005M PO_4 buffer (21)	In 2% proteose peptone (14)
Q_{O_2}	26.0	81.1	16.5	78.8
Cu. mm./hr./10^6 ciliates	140.2	433.0	133.7	633.0

oxygen consumption is a linear function of time over a period of three hours in all cases. Experiments were terminated at three hours.

It might be expected that exponential phase ciliates would show a higher rate of respiration than organisms in the stationary phase. This difference does appear, particularly in buffer suspensions.

DISCUSSION

Very little satisfactory work correlating population with size changes has been done with protozoa although there are good data on bacteria and yeasts. Henrici (1928) showed that cells of *Bacillus megatherium* increase in size during the lag period and up to the point at which population growth rate is maximum, whereupon they begin to decrease in size immediately. Huntington and Winslow (1937), in an examination of the size changes in *Escherichia coli, Salmonella gallinorum* and *Salmonella pullorum,* show that in all three there is an increase in size in the lag period and a decrease in size during the exponential period. Richards (1928)' has shown that yeast cells increase in size in the very early growth period and then decrease in size during the late exponential growth to a size comparable with that of the cells originally inoculated. The observations on protozoan populations have largely been made on

cultures contaminated by bacteria. Maupas in 1888 observed that individuals decreased in size during "senile degeneration" in old cultures. Calkins (1904) likewise showed that *Paramecium* decreased in size as the cultures died out. Woodruff's (1913) data on the growth of *Oxytricha fallax* can be roughly correlated with the growth phases. He observed that individuals were small during rapid growth and increased in size later. Peters (1921), on the other hand, noted that in cultures of *Colpidium colpoda* the population grew very slowly for nine to ten days during which time the individual size increased and remained large during the period of rapid growth which followed. Later the decrease in size was correlated with a decrease in population. Vieweger (1925) states that in cultures of *Colpidium colpoda* the body size decreased following the period of rapid exponential growth. His first measurements following the end of the exponential phase appear to have been made at six days, however.

All the work mentioned in the preceding paragraph was done with contaminated cultures and without complete data regarding the population curve being obtained. Bond (1933), using sterile cultures of *Tetrahymena geleii* (*Colpidium campylum*), and Lwoff (1932), using *Tetrahymena geleii* (*Glaucoma piriformis*), give data that indicate an increase in individual size following the exponential period. Their data concern total dry weight and total nitrogen change respectively. Dewey and Kidder (1940) give complete data on change in size in *Perispira ovum* fed on *Euglena gracilis*. They show an increase in size in the lag and early exponential phases. The size changes in *Perispira ovum* cannot be compared directly to those in *Tetrahymena geleii* since the increase in size in the former may be due largely to the accumulation of unassimilated *Euglena* in the individual ciliates. The subsequent decrease in size beginning in the late exponential period is correlated with a drop in the concentration of the food organism, *Euglena*. Any change in individual amount of *Perispira* protoplasm is obscured by the presence of masses of ingested but unassimilated *Euglena*.

The present investigations have brought out several points with regard to normal growth in populations of *Tetrahymena geleii*. The stationary phase of growth, so-called, has been found to be a truly stationary phase only so far as the numbers of individuals are concerned. It is seen that the number of fissions in this stage is very low while the population remains essentially static in number. This indicates that the individual organism has a much longer life in this phase and that there is very little death occurring. It is also seen that another process, that of individual growth, proceeds concurrently with that of population

growth until the end of the exponential phase whereupon it continues with the result that in the hours immediately following the onset of the stationary phase the organisms increase in size. It is thus apparent that the population curve taken alone is not a true representation of the growth of the culture.

Normal respiration data on *Tetrahymena geleii* is summarized in Table 5. M. Lwoff (1934) obtained the figure of 35 cu. mm. O_2/hr./mgm. dried organisms in peptone water using the Warburg method but does

TABLE 5

Comparative values of Q_{O_2} *for Tetrahymena geleii.*

Investigator	Approx. age of culture	Bacteria-free	Temp. °C.	Substrate present	Cu. mm. O_2/hr./10^6 organisms	Cu. mm. O_2/hr./mgm.
Peters (1929)[a]	?	Yes(?)	?	+	50–200	6.2–24.7[b]
M. Lwoff (1932)	?	Yes	22°	+	271.5[b]	35
Baker & Baumberger (1941)	48 hrs.	Yes	20°–22°	+	154[b]	19.01
Pitts (1932)	48 hrs.	No	24°	−	151	18.7[b]
R. H. Hall (1938–1941)	48 hrs.	Yes	19.8°	+	112.5	13.9[b]
Present investigator	50 hrs.	Yes	26.8°	−	133.7	16.5
Present investigator	50 hrs.	Yes	26.8°	+	632.5	77.7

[a] The ciliate used was called *Colpidium colpoda*. It may or may not have been *T. geleii*.

[b] These values are computed on the basis of the weight curve in Figure 2. 10^6 organisms equal 8.1 mgm. at 50 hrs.

not mention the age of the organisms used or the number of organisms per mgm. of dry weight. Pitts' (1932) figure was obtained using a non-nutrient suspension that had grown on bacteria for 48 hours previously. Baker and Baumberger (1941) used a dropping mercury electrode and a suspension of *Tetrahymena geleii* in yeast autolysate dextrose. They did not determine the dry weight but calculated it on the basis of 50 μ being the average length of an organism and the dry weight being about 15 per cent of the wet weight. R. H. Hall (1938, 1941) apparently used M/15 Sørensen phosphate and 0.5 M Clark and Lubs biphthalate buffers for his suspensions. It was found in the course of these investigations that M/50 Sørensen phosphate buffer depressed the respiration rate markedly and that even M/100 phosphate had a slight inhibitory effect.

A number of factors may have contributed to the different results shown in Table 5. The organisms in the several cases may not have been of comparable age and weight and there may have been consider-

able strain differences. Likewise there may have been some errors due to bacteria or the products of bacterial metabolism.

The age of 48 hours is significant only when the length of the exponential phase is known likewise, so the figures given in Table 5 on the age of the cultures are for the most part only rough approximations of the actual age of the ciliates in terms of the population curve. Strain differences are known to exist and may exert an influence on the rate of respiratory activity. The possible toxic or beneficial action of bacteria is well known. There may have been errors in the determinations by Baker and Baumberger due to their approximations regarding volume and weight of the ciliate. The inhibitory action of phosphate buffer solutions previously mentioned may vitiate Hall's figure. The different substrates used in the various determinations are undoubtedly influencing factors. The most probable reason for the high Q_{O_2} obtained in the present investigation is the utilization of a nontoxic concentration of buffer, and a substrate that is comparatively concentrated and to which the organisms have been thoroughly acclimated.

In comparing the rate of respiration in exponential and stationary phase ciliates in buffer suspensions on the basis of number of individuals, the values obtained were 140.2 cu. mm./hr./10^6 organisms and 133.7 cu. mm./hr./10^6 organisms respectively. When buffered 2 per cent proteose peptone suspensions were used the rate per 10^6 organisms is higher in the *stationary* phase. This situation is clarified when reference is made to Figure 2 and Table 4 and illustrates the errors that may be involved in comparing respiration rates on a population basis alone. The Q_{O_2} is defined as the cu. mm. O_2/hr./mgm. dry weight of respiring material. On this basis the Q_{O_2} of exponential phase ciliates is 26.2 since the weight of 10^6 organisms is 5.33 mgm. As the weight of the stationary phase ciliates increases rapidly following the end of the exponential period, the weight of 10^6 organisms at 50 hours is 8.10 mgm. This gives a Q_{O_2} of 16.5 or 63 per cent of the rate found in the exponential phase. This emphasizes the point that respiration rates should be compared on the per-unit-of-protoplasm basis rather than on the basis of individual organisms. The relatively greater increase in Q_{O_2} in stationary phase organisms when in proteose peptone suspensions suggests that the conditions under which measurements are made are much more like those of stationary phase cultures than those of exponential phase cultures due to the high concentration of organisms. External conditions may be limiting factors in the respiration of exponential phase organisms under these conditions.

In the matter of decreasing oxygen consumption with increasing age of the culture there is unanimity. Peters (1929) reports a drop from a Q_{O_2} of 200 in growing cultures of *Colpidium colpoda* to one of 50 in old cultures. Wachendorff (1912) reports a like decrease in the respiration of *C. colpoda* within 30 days from the time the culture was started. Baker and Baumberger (1941) show a decrease in *Tetrahymena geleii* suspensions beginning in the last exponential phase and dropping to approximately one-third the highest value obtained during that period, in from three to seven days. Hutchens (1941b) has found the same phenomenon in *Chilomonas paramecium* within a period of 72 hours. In the present investigations, likewise, a decrease in oxygen consumption has been found in the population phases following the exponential period of growth.

Summary

1. The relation of cell growth to population growth in bacteria-free cultures of *Tetrahymena geleii* has been examined.

2. Cell growth has been found to proceed independently after the onset of the stationary period of population growth.

3. The ratio of fissions occurring to the population concentration has been determined and described.

4. The normal Q_{O_2} of exponential and stationary phase ciliates in phosphate buffer and in proteose peptone has been determined.

5. A significant difference in the respiratory rates of organisms from the two phases has been shown.

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THE SEXUAL CYCLE OF THE SHIPWORM, TEREDO NAVALIS

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In an earlier paper Grave and Smith (1936) published results of an extensive study of adult *Teredo* (over-wintering *Teredo*) which showed conclusively that numerous individuals change sex, not only from male to female but also from female to male, during the course of the breeding season. That this is a part of the normal sexual cycle of *Teredo* is shown by three types of evidence: a study of stained paraffine sections; teased gonads of living animals; and a tabulation of sex ratios.

During the past summer a similar study was made of young shipworms of the current season in the region of Woods Hole with comparable and confirmatory results. Early in the summer *Teredo* traps in the form of wooden crates made of lobster pot lath or of fir boards one-half inch in thickness and approximately three inches wide were placed in sea water known to be infested with *Teredo* larvae. By examining slats from these traps at frequent intervals during the summer it was possible to follow the course and rate of development of the young animals. More important for the purpose of this study, it was possible to determine the age at which they first became sexually mature and to tabulate sex ratios at various stages of development consecutively. Table I therefore shows the approximate age of the animals examined, the increase in size with age or rate of development, and the relative numbers of the sexes from week to week during the breeding season.[1]

[1] Sexually mature males are easily recognized. When removed from their burrows they expel active spermatozoa visible under a low power of the microscope. In practice it was found necessary to place each individual in a separate dish and the test of maleness was the presence of abundant active sperm in the surrounding water. As a further test the body wall was torn with dissecting instruments and a portion of the gonad removed and examined microscopically to determine its character, whether male, female, or sexually immature. If male, abundant active spermatozoa were released; if female the gonad wall showed a complete layer of large yolky oögonia. Sexually mature females are characterized by oöcytes which readily separate from the gonad wall when teased with needles. It should be noted that most males have a few or numerous small oögonia without yolk (Grave and Smith, 1936, Plate II, Figures 5 and 6). The entire gonad of both male and female with its ducts resembles a bunch of grapes except that the terminal alveoli which produce the germ cells are not spherical but elongated, sinuous, and somewhat intertwined.

The points of chief interest are that some of the young animals became sexually mature at 40 or 42 days when they measured from 27 to 30 mm., and that males and females appeared simultaneously. This rate is the most rapid attained at Woods Hole. Larvae which metamorphose late in June or early in July require seven or even eight weeks to reach sexual maturity.

Although only a few individuals are sexually mature at this early stage the data show that males and females appear simultaneously. The data of Table I dealing with somewhat later stages of growth show that throughout the breeding season there is no great preponderance of one sex over the other. It is only toward the end of the first breeding season that the females appear in considerable excess and that excess never approaches 90 per cent either in young or in over-wintering animals. This is in substantial agreement with other tabulations of sex ratios in *Teredo* (Grave, 1928; Grave and Smith, 1936; Coe, 1936).

Statistical tabulations are always open to criticism because of the unavoidable element of analysis and of conscious or unconscious manipulation. It is therefore desirable to bolster such studies, valuable as they may be, with facts or observations not subject to more than one interpretation. If it can be shown that female teredos are changing to males during the entire breeding season it should be evident that the population could not become 90 per cent or exclusively female in adult life as required by the theory of protandry. The following examples which are described in detail are of frequent occurrence. They constitute the chief evidence that this sex inversion is a normal part of the sexual cycle of *Teredo*.

Evidence of Change of Sex from Female to Male

There is dependable evidence that *Teredo* may and does change sex during the breeding season. It is not in question that a sexually mature male may change to a female. That individual teredos also change from female to male is supported by observations of specific cases, three of which are presented. These cases, it is believed, are subject to no other interpretation. The so-called hermaphroditic teredos are not really hermaphroditic but are transition stages of brief duration of individuals in process of changing from male to female or from female to male so that the male and female phases may overlap for a day or two. This point cannot be too strongly emphasized and any investigator who studies *Teredo* extensively is likely to come to this conclusion. Nearly all individuals are either in the male phase exclusively, or exclusively female and not both. The examples selected will serve to corroborate this interpretation of hermaphrodites.

Case I.—A young *Teredo* of the current season two months old was carrying large numbers of veliger larvae in its brood pouches. From their color the veligers were judged to be ten days or two weeks old. In other words, this individual had been a female when the eggs producing this brood of larvae had been spawned. An examination of its gonad showed it to be a testis containing great numbers of active spermatozoa and spermatogenous tissue. In the wall of the gonad there were also a few small quiescent yolkless oögonia. This is a characteristic of *Teredo* in the active male phase (Grave and Smith, 1936, Plate II, Figures 5 and 6). It is noteworthy also that this was a young *Teredo* which changed from female to male after a single spawning. It was not old enough to have spawned twice since it could not have become sexually mature before six weeks of age. Point is made of this case because it shows that females are changing to males during the first season as well as in the adults which were described in detail and with illustrating photographs in a former paper (Grave and Smith, 1936).

Among cases of sex inversion from male to female observed in young teredos during the past summer, one was two months old, another ten weeks. Since they are not otherwise significant to this study they are not described and the reader is referred to published descriptions and photographs (Grave and Smith, 1936, Plate II, Figure 4) which show how a male changing to a female may be recognized.

Case II.—During the present study of adult shipworms numerous cases of sex inversion from female to male were observed and examples were selected for description. One of these showed the following characteristics: It contained numerous veliger larvae and also abundant cleaving eggs in its gills, showing that it had spawned twice as a female. Its gonad contained abundant active sperm and no oögonia. There can be no doubt that this individual had recently changed sex from female to male.

Case III.—Stained paraffine sections prepared for the study of gonads of adult shipworms showed all degrees of maleness and femaleness, including true males. From hundreds of preparations one individual changing from female to male was selected for description, a photomicrograph being made to record its character. Each of the alveoli of the gonad contained one or more oöcytes free in the lumen while the parietal wall was clearly composed of spermatogenous tissue, including spermatogonia, spermatocytes and spermatids. This is clearly the condition of the gonad because abundant spermatozoa were already free in the lumen surrounding the free oöcytes. This individual might be called an hermaphrodite but no oögonia were present in the gonad wall

and as soon as the floating oöcytes had passed out it would have become exclusively male. This photograph can be interpreted in no other way than that the individual was changing from female to male (Grave and Smith, 1936, Plate I, Figure 3).

It has been the purpose of the foregoing account to present positive evidence from the study of living animals and paraffine sections that teredos normally and regularly change sex, beginning shortly after they

TABLE I

Rate of growth and relative numbers of the sexes of young Teredo taken from Teredo traps composed of small fir boards placed in sea water at Woods Hole, July 1, 1941

1	2	3	4	5	6	7	8
Date of collection	Approximate age	Size of burrows	Males	Females with eggs	Females with embryos	Hermaphroditic	Not spawning
Aug. 8	38 days	8–10 mm.					5
" 9	39 "	8–12 "					10
" 10	40 "	16–20 "					6
" 11	41 "	16–22 "					10
" 12	42 "	20–22 "					2
" 19	49 "	28–35 "	4	1			1
" 19	49 "	40–43 "	1	2	2		
" 21	51 "	44–52 "	4	1			
" 22	52 "	35–39 "	2	1			1
" 22	52 "	40–60 "	5	6	7		
" 23	53 "	40–62 "	5	3		1	
" 28	58 "	40–45 "	6	2	1	1	1
" 28	58 "	50–70 "	4	4	8	2	1
Sept. 10	72 "	60–85 "	4	2	16		10
" 13	75 "	50–75 "	2	6	1		2
" 13	75 "	80–130 "	14	8	14	1	10

Table I, when read from top to bottom, shows in column 1 the successive dates of collection of *Teredo* from crates exposed to sea water on July 1; column 2 gives the maximum age of the specimens collected (by selecting only the largest specimens it may be assumed that they are approximately as old in days from metamorphosis as indicated in column 2); the size of the burrows as shown in column 3 is considered to be an accurate measure of size attained, since the animal in life exactly fills the burrow when relaxed; columns 4, 5 and 6 show the relative numbers of males and females found on successive collections; column 7 includes a small number of individuals whose gonads contained both active sperm and fully developed yolky oöcytes; column 8 includes all individuals which contained neither active sperm nor recognizable oöcytes which would indicate an approach to sexual maturity. However, the last three items of column 8 were dead animals which had disintegrated beyond recognition of sex, indicating a high mortality after September 1.

Table I not only shows the relative numbers of the sexes at successive periods of the breeding season but also indicates that teredos in the progonad stage are not sexually mature. (See first five items of Table I.) This conclusion is based upon the fact that no active sperm and no eggs are expelled during this early period.

become sexually mature and continuing throughout the remainder of their lives. This inversion of sex is in both directions, from male to female and from female to male. It has been shown (Grave and Smith, 1936) that females change to males not only at the opening of the second breeding season, as described by Coe, 1934, but throughout the breeding season of adult or over-wintering teredos.

The investigations of the past summer, reported in this paper, show that the inversion of sex begins in animals only two months old and continues thereafter to be a part of sexual history of the species. It is clear therefore that if there is at all times a considerable inversion of females to males the sexual cycle of this species could not be described as overwhelmingly male in young animals and equally overwhelmingly female in mature animals. This type of sexual cycle has been attributed to *Teredo* and referred to as protandric. This study shows that both sexual changes occur throughout the breeding season, and consequently *Teredo* resembles the California oyster rather than *Crepidula,* both of which have been fully described by Coe. This contribution is considered important not merely because it adds a point to scientific accuracy but because of its bearing upon the problem of sex determination. Scientific literature dealing with sex determination when considered as a whole, especially that dealing with insects, mammals, and molluscs, is confused and it is idle to talk about a mechanism of sex determination. It is obviously more difficult to account for a mechanism of sex inheritance in an animal that changes sex three or four times than in one that is male when young and female at maturity (protandric). If investigators will keep facts clearly separated from interpretation and use principally either photographs or camera lucida drawings for illustration instead of diagrams, some future biological theorist may be able to unify all of the numerous and diverse contributions to the study of sex and sex inheritance. Sex changes in molluscs have been induced or hastened by changes in the environment of various kinds but internal factors are obscure. This has been shown to be true, especially by the experiments of Coe on *Crepidula.*

The Progonad and the Sexual Cycle

This account to this point has not involved the very early stages in the development of *Teredo,* which precede sexual maturity. Young shipworms measuring from 10 to 20 millimeters and estimated to be not more than four to five weeks of age were fixed, sectioned and stained for microscopic study of the progonad.

At an early stage of development the gonad of *Teredo* consists of a solid mass of undifferentiated cells lying on either side of the digestive tract. In the process of further development it becomes a branching system of tubules with walls several cells in thickness but as yet indistinguishable as to sex. Following upon this, in some individuals, certain cells enlarge and become recognizable as oögonia. These large cells arise in the midst of more numerous smaller cells which still appear to be undifferentiated. Whether the latter are actually undifferentiated or are potentially spermatogonia and oögonia is difficult to make out. In any case individuals which have large oögonia in the gonad wall at this early stage of development are destined to become females, either purely female or with some spermatogonia among the developing oögonia. These oögonia early take on a deposit of yolk and the spermatogonia, if present, become aborted without functioning, and the gonad becomes an ovary. Other individuals are destined to differentiate into males whose gonads appear to be purely male or they may contain small yolkless oögonia which remain quiescent for the time being. These individuals produce abundant active sperm and are functional males. Thus the cytological evidence confirms results obtained by the study of living animals to the effect that males and females develop simultaneously. Both males and females appear in considerable numbers within eight weeks of metamorphosis. One gets the impression that the balance between femaleness and maleness in *Teredo* is not rigidly fixed at any period of its development so that males may become females and females become males with slight alteration of internal or external conditions not as yet understood. The young gonad appears to be indifferent as to sex and as a result all gradations between maleness and femaleness appear in the gonad itself.

Although the progonad does not produce active spermatozoa it, in a sense, may be considered a male phase of brief duration. This would be true only if the undifferentiated cells in the young gonad surrounding the developing oögonia are properly considered to be spermatogonia. Otherwise approximately half of the young teredos are actually female. In any case numerous individuals differentiate first as functional females and do not produce active sperm. It has been estimated that about half of the individuals are females from the first appearance of germ cells in the young gonad.

Discussion and Conclusions

The sexual cycle of *Teredo* may be regarded as beginning with the differentiation of sex. By the end of eight weeks nearly all individuals are sexually mature and the inversion of sex from males to females and

from females to males begins. This change of sex from female to male prevents any *Teredo* population from becoming overwhelmingly of one sex either at maturity or at the beginning of sexual life. Both sexes are well represented at the end of eight weeks of age and because of the inversion of sex in both directions both sexes remain well represented in all tabulations of sex ratios that have been made. It should be clear therefore that the observed changes from female to male during the breeding season constitute part of the sexual cycle in this species.

It remains only to point out that the term "protandric female" as used by Coe (Coe, 1933) is synonymous with the term "functional male" or "bisexual male" as used by Grave and Smith, 1936, and in this paper. The writer prefers to regard such individuals as males since they constitute most if not all of the existing males, while Coe considers them potential females in anticipation of their change to females as a part of a protandric sexual cycle. If they actually do change to females without any counterbalancing change of females to males the population would ultimately become wholly or predominantly female. Since it has been shown that females do change to males there is necessarily a recognition of the fact that the general population remains always considerably male, and the conception of a protandric sexual cycle should be modified to recognize this feature, emphasizing the fact that there are changes of sex in both directions during the entire breeding season as well as at its beginning.

Since the completion of this manuscript, September 20, 1941, an article by Coe (1941) has appeared. As in former papers, he insists on the essentially protandric nature of the sexual cycle of *Teredo navalis*. His facts and mine are similar except that I do not consider the progonad as 90 per cent male. The point made in this paper, considered to be its chief contribution, is that the changes in sex during the breeding season are not exclusively from male to female as required for a protandric sexual cycle but also from female to male. It has been pointed out that this latter sex inversion necessarily changes the whole concept of the sexual cycle and accounts satisfactorily for the high percentage of males present during the entire breeding season. Coe regards the succession of sex changes from male to female as a function of age and he therefore considers the general population as being composed of animals of many different ages, thus accounting for the persistence of numerous males at all times during the entire course of the breeding season.

In a former paper the writer showed that all over-wintering teredos, even those that are too small to be detected in the wood during the winter months, become sexually mature before the end of June (Grave, 1928).

The over-wintering teredos are therefore not just becoming sexually mature all summer. It would seem also that the observed changes from female to male which take place in both young and over-wintering teredos should be considered to be an essential part of the sexual cycle. It is clear also that a considerable number of individuals function first as females and these have no primary functional male phase. *Teredo navalis,* therefore, according to this interpretation, is not essentially or principally protandric.

I wish to express my obligation to Dr. J. A. Smith for assistance in embedding and sectioning material and for criticizing the manuscript.

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THE INACTIVATION OF FERTILIZIN AND ITS CONVERSION TO THE "UNIVALENT" FORM BY X-RAYS AND ULTRAVIOLET LIGHT

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Recently Tyler (1941) has shown that the substance known as fertilizin which is obtained from the eggs of certain marine invertebrates and which has the property of agglutinating species sperm, may exist in non-agglutinating form. Thus, fertilizin (egg water) of *Strongylocentrotus purpuratus,* when subjected to appropriate heating or enzymatic digestion, lost its ability to agglutinate, but retained its ability to combine with the sperm. Tyler has proposed the term, "univalent fertilizin," for such non-agglutinating substances. They are readily demonstrable since their addition to sperm inhibits subsequent agglutination by ordinary fertilizin of the same species.

Several workers have shown that the sperm agglutinating power of *Arbacia* egg water can be destroyed by X-rays. Richards and Woodward (1915) reported an initial rise and subsequent fall in sperm agglutinin titer with prolonged irradiation. More recently, Evans (1940), and Evans, Beams and Smith (1941) have shown that the egg jelly of *Arbacia* is dissolved by X-rays and that irradiated egg water will not give the Janus green reaction (Harvey, 1939) nor will it agglutinate species sperm.

It appeared possible that univalent fertilizin might be produced during treatment of fertilizin with X-rays. The present work was done primarily to examine this possibility. The results show that it does indeed occur. In the course of the work further quantitative data were obtained on the X-ray inactivation of fertilizin in terms of Roentgen units, fertilizin concentration and sperm concentration. In addition the possible action of another type of radiation, namely ultraviolet light, in inactivating fertilizin and producing univalent fertilizin was investigated. Here, also, the results were positive.

Material and Methods

The sea urchin, *Arbacia punctulata,* was used exclusively in the experiments with X-radiation, while *Strongylocentrotus purpuratus* was

used in the experiments with ultraviolet light. Eggs and sperm were obtained by opening the animals and allowing them to shed into stender dishes. Fertilizin was prepared by removing the supernatant sea water from a suspension of eggs which had stood overnight in the refrigerator, or by extracting eggs in acid sea water (Tyler and Fox, 1940) and readjusting the pH of the extract. All sperm suspensions were made from "dry sperm" (sperm collected from the gonopores in dry stender dishes). The concentrations of sperm are given as percentages of this dry sperm. The presence or absence of an agglutination reaction was determined by microscopic examination two to four seconds after mixing the solutions and sperm suspensions.

X-radiation in all cases was done in waxed paper cups at 5,600*r*/minute.

Ultraviolet radiation was given in open stender dishes. Controls were run in open dishes placed at the same distance (4.5 cm.) from the radiation source, but screened by a filter (Noviol "C"). A quartz-mercury arc (tube dimensions: 56 × 0.8 cm., power supply: a 15,000 volt, 30 milliampere transformer) was used as the ultraviolet light source.

Experiments

Inactivation of Fertilizin by X-radiation

In a typical experiment, a sample of egg water obtained as the supernatant from 50 cc. of eggs was divided into five equal parts. One part was kept as control and the other four samples were irradiated as indicated in Table I. After irradiation the agglutinating titer of each sample

Table I

Destruction of agglutinating power of Arbacia egg water by X-radiation

r units	Fertilizin titer	Per cent inactivation
0	2,048	—
2,800	1,024	50
5,600	256	87.5
16,800	2 (?)	99.9
28,000	0	100

was determined by serially diluting the samples in ½ dilutions with sea water and testing each dilution with an equal amount of 0.5 per cent sperm suspension. Titers are given as the greatest dilution at which agglutination is still microscopically perceptible. The units are, there-

fore, roughly 20 to 40 times greater than those employed by Tyler and Fox (1940).

As shown in the table, inactivation of this sample of fertilizin (titer 2,048) is practically complete with irradiation above 16,800*r*. Two other experiments gave similar results.

Richards and Woodward gave no information concerning the output of their X-ray apparatus. Since they used a very weak fertilizin solution and noted only a slight fall in agglutinin titer after 7.5 minutes of irradiation, it may be concluded that they used relatively low X-ray doses. Evans, Beams and Smith (1941) irradiated samples of *Arbacia* egg jelly (egg suspensions?) with 61,000*r* and 122,000*r*. The former sample gave light agglutination and the latter gave none. These values are in agreement with those presented here, assuming that dilute test sperm suspensions were used, since the same workers have shown, in *Arbacia*, that X-ray doses of 15,000*r* completely remove the jelly, which is identical with or contains the fertilizin (Tyler, 1940, 1941). Thus, an irradiated egg suspension is equivalent to an irradiated fertilizin solution of high titer.

Production of Univalent Fertilizin by X-Rays

As is well known (Lillie, 1919; Tyler, 1940), the agglutination of sea urchin sperm by egg water is a spontaneously reversible phenomenon. Furthermore, sperm which have been agglutinated by a sufficiently strong egg water will not, after reversal, reagglutinate upon further addition of fertilizin. Theoretically, sperm treated with fertilizin that had been converted to the univalent form should be equivalent to sperm that had reversed and so no agglutination should occur upon subsequent addition of normal fertilizin.

The sperm agglutinin titer of any fertilizin sample may be determined by serially diluting the sample and testing for agglutination with constant amounts of a sperm suspension, as previously outlined. If, after reversal of this agglutination, equal amounts of a test (normal) egg water are added to each dilution and the greatest dilution at which no agglutination occurs is recorded, a quantitative measure of the agglutination inhibiting power of the sample is obtained, which may be termed the inhibition titer of the sample. When both the agglutinin and inhibition titers of an irradiated sample and its unirradiated control are determined, using the same sperm suspension and test egg water solution, the formation of any univalent fertilizin through the treatment may be detected by a comparison of these values. It is thus not necessary to inactivate the fertilizin completely to test for the univalent form. This is desirable, in

that excessive irradiation might result in complete destruction of the fertilizin.

A strong fertilizin solution was prepared by extracting eggs in slightly acid sea water (pH 4.5). By this treatment 105 cc. of egg suspension gave 65 cc. of egg water and 40 cc. of jellyless eggs. After readjusting the solution to pH 8, a 5 cc. sample was removed and irradiated for five minutes at 5,600*r*/minute. Two drops of the irradiated solution and two drops of the control were diluted serially in tenfold steps with sea water, two drops of a 1 per cent sperm suspension were added to each dilution, and the dishes were allowed to stand until the agglutination in the undiluted control had reversed. Two drops of 1/10 dilution of the original control fertilizin were added and the dishes examined for agglutination.

TABLE II

Ability of X-rayed Arbacia fertilizin to react with sperm and thus inhibit subsequent agglutination of this sperm by test fertilizin

	Agglutinin titer	Agglutination after reversal of control upon addition of a test egg water to undiluted samples	Inhibition titer (Reciprocal of dilution of egg water at which test egg water first gave agglutination)
Control egg water.....	100,000	Negative	1,000
Irradiated egg water...	100	Negative	1,000
1,000 fold dilution of control egg water....	100	Positive	0

The irradiated egg water was found to have about 1/1,000 the agglutinin titer of the control. However, it was found to be just as effective as the control solution in preventing subsequent agglutination. The quantitative data are given in Table II.

It is of interest that no precipitate which might be attributed to this radiation was seen to form in this or any of the other X-rayed fertilizin solutions, even after prolonged exposure.

Inactivation of Strongylocentrotus Fertilizin by Ultraviolet Light

A stock solution of *Strongylocentrotus purpuratus* fertilizin was made up by extracting, with acid (pH 3) sea water a concentrated suspension of shed eggs, centrifuging and adjusting the supernatant to pH 8. Two 4 cc. samples were removed from this stock solution. One was irradiated and the other used as a control as previously described.

At various intervals of time equal portions of solution (four drops) were removed from the irradiated and control samples for titration of

sperm agglutinin activity. Titrations were made by adding equal amounts (two drops) of 1 per cent sperm suspension to equal amounts of the fertilizin diluted serially in ½ dilution steps. The titer, as in the case of *Arbacia*, was taken as the greatest dilution at which agglutination could be observed in two to four seconds under the microscope. Since the same 1 per cent sperm suspension, pipettes, etc., were used throughout, the titrations, as presented in Table III, are directly comparable. The results are typical of several experiments.

TABLE III

Destruction of agglutinating power of S. purpuratus fertilizin by ultraviolet light

Duration of irradiation	Agglutinin titer	Per cent inactivation
0	16,384[1]	—
60 min.	1,024	93.8%
90 "	256	98.4%
124 "	16	99.9%
154 "	0	100.0%
349 "	0	100.0%

[1] Average of the five controls, none of which differed more than one dilution step from this value.

As the data indicate, complete inactivation of the sperm-agglutinating property of this fertilizin solution was attained in 2.5 hours by the ultraviolet light source. Since both the controls and irradiated samples were allowed free access to air and were the same distance from the radiation source, evaporation and effect of ozone are ruled out as causal agents. Thermometers placed in the position of the control and irradiated samples indicated at most a 2° C. rise above room temperature, but never a difference of more than 1° C. in the two positions, so the inactivation found is attributed to the radiation from the arc.

Production of Univalent Fertilizin by Ultraviolet Light

All irradiated and control fertilizin samples recorded in Table III were tested for univalent fertilizin by adding one drop of a test fertilizin solution (stock solution diluted to 20 per cent) to each dish after reversal had occurred in the undiluted controls. As before, the inhibition titer is that dilution of the original egg water (irradiated or control) at which agglutination was first observed upon addition of the test fertilizin. Table IV gives the data for the sample irradiated for 349 minutes. This result is similar to that obtained with the other four samples which received lower radiation doses.

The data show less inhibition (one dilution step) for the irradiated than for the control fertilizin. The difference is within the error of the method (it was found in the other four determinations as well), and may be attributed to inaccuracies in making up the dilutions, to partial inactivation of the specific combining groups of the fertilizin by the ultraviolet treatment, or more probably to incomplete reversal of the initial

TABLE IV

Ability of ultraviolet light treated S. purpuratus fertilizin to react with sperm and thus inhibit subsequent agglutination of this sperm by test fertilizin

	Agglutinin titer	Agglutination after reversal of control upon addition of test fertilizin to undiluted samples	Inhibition titer (Reciprocal of dilution of egg water at which test egg water first gave agglutination)
Control for sample irradiated 349 min.....	16,384	Negative	8
Egg water irradiated 349 min.....	0	Negative	4

agglutination in the control. Microscopically "complete reversal" is an ideal condition seldom attained with strong egg water. Any small unreversed clumps of sperm obscure a faint reaction by the test egg water.

Experiments on the fertilizin of the sea urchin *Lytechinus anamesis* showed that it also lost its sperm agglutinating power and became univalent when treated with ultraviolet light.

DISCUSSION

An attempt has been made to give standardized data on the X-ray inactivation of *Arbacia* fertilizin and the ultraviolet light inactivation of *S. purpuratus* fertilizin. The limitations involved in the use of "dry" sperm as a standard for sperm concentration are fully appreciated. The concentration of spermatozoa in dry sperm varies from animal to animal and with the method used in obtaining the sperm. The standard is, however, a convenient one, and has been recognized by wide application. It is accurate enough to justify its use here.

Tyler (1941) has suggested that the well known reversal of sperm agglutination in sea urchins results from a splitting of the fertilizin molecule, leaving univalent fragments attached to the sperm; and that non-agglutinating fertilizin obtained through heating or enzymatic digestion likewise involves a splitting of the molecule to form univalent

fragments. The initial rise in agglutinin titer recorded by Richards and Woodward (1915) for X-rayed fertilizin, and by Tyler (1941) for heat or enzyme treated fertilizin, are interpreted as the initial step—a splitting of the highly multivalent molecule into two or more smaller multivalent fragments—in the production of univalent fertilizin. This initial rise in agglutinin titer was not observed in the present work, although it might have been found with lower dosages.

It is shown here that fertilizin treated with X-rays or ultraviolet light loses its sperm-agglutinating power, but retains all or nearly all of its ability to combine with sperm. This indicates that a destruction of all but one sperm-combining group per molecule of fertilizin is probably not the mechanism of this inactivation. On the contrary, the data favor rather strongly Tyler's view that the original multivalent molecule is split into fragments containing but one combining group per molecular fragment.

Heat, ultraviolet light, and X-rays are denaturating agents (Clarke, 1936; Arnow, 1936), and since fertilizin is probably a protein (Tyler and Fox, 1940) it is possible that univalent fertilizin is formed as a result of incomplete denaturation of this substance. On the basis of Tyler's explanation of univalence, this would imply that in denaturation there is initially a splitting of the molecule. Such an effect has been recorded by Svedberg and Brohudt (1938) for *Helix* haemocyanin. When solutions of this material were irradiated with ultraviolet light at pH 7.4 a splitting into half and into smaller molecules occurred, followed, with prolonged irradiation, by complete coagulation. Bawden and Pirie (1937) showed that heat denaturation of the tobacco mosaic virus protein resulted in a splitting off of nucleic acid. However, a number of simpler proteins undergo little if any change in molecular weight as a result of denaturation. Sanigar, Krejci and Kraemer (1939) report no change in the absorption spectrum or sedimentation behavior of purified serum albumen after X-radiation, although treatment with ultraviolet light resulted in the appearance of substances of low molecular weight. No cleavage of the egg albumen molecule was found by Fricke (1938) through the action of X-rays, although heating caused a slight hydrolysis, the effect being greater after irradiation. Likewise, Mirsky (1938) obtained no decrease in molecular weight in the heat or acid denaturation of trypsin. Obviously, no general rule may be formulated from the work on denaturation which may be applied to the inactivation of the agglutinating power of fertilizin and the simultaneous appearance of the univalent material but Tyler's view of a fragmentation of the fertilizin molecule is supported by some of the above work and is favored here as the simplest explanation for the results obtained.

Summary

1. The X-ray inactivation of *Arbacia punctulata* fertilizin reported by Richards and Woodward (1915) and by Evans, Beams and Smith (1941) is confirmed. Further data on this inactivation are presented in terms of Roentgen units, fertilizin concentration and sperm concentration.

2. It is shown that ultraviolet light is effective in the inactivation of *Strongylocentrotus purpuratus* and *Lytechinus anamesis* fertilizin.

3. *Arbacia* fertilizin which had been almost completely inactivated by X-rays, as judged by its ability to agglutinate sperm, lost none of its power to react with sperm. Thus, sperm treated with this fertilizin showed the same resistance to subsequent agglutination by untreated fertilizin as did sperm which had reversed after treatment with unirradiated (control) fertilizin.

4. *Strongylocentrotus* and *Lytechinus* fertilizins whose sperm agglutinating powers had been completely destroyed by ultraviolet light likewise retained their original sperm combining powers, as indicated by their ability to render sperm non-reactive to untreated fertilizin.

5. Tyler's (1941) view that fertilizin may be converted to a non-agglutinating, "univalent" form by splitting the fertilizin molecule into fragments, each having but one reacting group, is considered the most reasonable explanation for the results.

I am grateful to Dr. Albert Tyler for suggesting the problem, to Dr. T. C. Evans for much helpful advice, and to Dr. E. P. Little for X-raying the *Arbacia* material.

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DO SPERMATOZOA PENETRATE THE MEMBRANE OF SELF-INSEMINATED EGGS OF CIONA AND STYELA?

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In 1935–36 I made many preparations (sections) of unfertilized eggs, and self- and cross-fertilized eggs of *Ciona* to find out whether spermatozoa do or do not penetrate the membrane of their own eggs. The main difficulty in reaching a decision was that the test-cells are so filled with granular materials that it is generally difficult to distinguish these, when stained, from the head of the very small spermatozoön. Nevertheless certain stains were found that could be de-stained to a point where the color of the particles in the test cells was removed leaving the stain in the heads of the sperms. Iron haematoxylin (half an hour) followed by thorough extraction gave good slides. More striking contrasts were obtained in eggs (killed in Bouin or in Carnoy solutions), sectioned, and stained in basic fuchsin (15 minutes); followed by picro-indigo carmine (six minutes), and washed in tap water.

The results showed that the spermatozoa do not as a rule pass the membrane of the eggs of the same individual. In other words, the block to self-fertilization is in the membrane.

During the winter of 1941–42 I have studied the problem again. In addition to the stains mentioned above, Ehrlich's haematoxylin (seven minutes) extracted in acid alcohol (half a minute or less), gave excellent preparations, as also did acetic-orcein (LaCour) followed in some cases by a fast green counterstain. For the study of the sections I had a better set-up than before, especially better illumination. Practically all the sections were examined in collaboration with Dr. Jack Schultz. Each case was examined by both of us. I am grateful to him for help and critical examinations.

Ciona Intestinalis

A few spermatozoa are as a rule found inside the membrane of cross-fertilized eggs, but only one or two in a section, some of them against the surface of the egg, others among the test cells. The sperm are stained

deep red after orcein or haematoxylin. It is difficult to determine whether any of the sperms are inside the test cells or merely between them; the best evidence seems in favor of the latter view. In *Ciona* the test cells form practically a continuous covering around the egg, and it might seem that the sperm are obliged to pass through them to reach the surface of the egg, but the test cells are loosely held together and it may be that sperms can pass between them. The test cells are green after the orcein and fast green, and the sperm red. They are unstained after the haematoxylin if properly extracted. The red-stained heads of the sperms, both outside the membrane and inside it, are strongly contrasted with the contents of the test cells.

The eggs that have been carefully removed from the oviduct, with no sperm added, show in sections, deeply stained particles and strands in the test cells unless the test cells are thoroughly de-stained, or are very little stained. These particles are of a different color from the sperm (orcein and fast green). The contents of the test cells are in these respects exactly like those in the self- and cross-fertilized eggs.

The selfed eggs are like the unfertilized eggs. In one or two cases a sperm has been found inside the membrane. Inasmuch as selfing may occur on a small scale such exceptional cases may be expected to occur, even in sets where no later cleavage happened; for, unless a sperm penetrated the membrane near the antipole, it might not enter the egg.

Certain precautions have to be taken with the sections of these selfed eggs. Occasionally one or more outside sperms may be dragged by the section-knife across the surface of the egg on one side. They will lie above or below the cut surface of the egg and, as a rule, there is no difficulty in detecting their origin. Slight folding of the edge of the section may give the appearance of sperm inside the egg, but such displacements are obvious.

Sections of cross-fertilized eggs show that spermatozoa may penetrate the membrane at all points of the surface, and not simply at the antipole where they also enter to fertilize the eggs. In exceptional cases it is possible that sperms may get in at other points of the surface of the egg before those that enter at or near the antipole, and if they do they may possibly enter the egg, but I have found no evidence of this. If they do, it is possible that abnormal cleavage or development would take place, but I have no grounds for such an assumption.

Styela (Cynthia) partita

At the end of the summer of 1941, five sets of eggs of *S. partita* were preserved. Records of the behavior of the remainder of the same eggs were made. The eggs were taken from the dishes in which they had

been ejaculated (along with the sperm) after 10 to 20 minutes. In some of these cases cross-fertilizations were also made to test the functioning of the sperm, and as a control of the selfed eggs. The sections were stained either in acetic orcein or in haematoxylin (Ehrlich).

1. Eggs and sperm were set free at 8 P.M. Some eggs were later preserved. None of the left-overs segmented, nor were embryos present the next morning. The stained selfed eggs showed no sperm inside the membrane, but stained sperm outside.

2. None of the selfed eggs was observed to segment, but next morning some normal tadpoles were present, presumably from eggs that cleaved late. The stained sections showed a few sperm inside the membrane both in the orcein and in the haematoxylin preparations. Some of the same eggs crossed to sperm of number (1) gave some embryos the next morning, showing that the sperm of (1) was "good."

3. None of the selfed eggs segmented. No sperms were found inside the membrane in the stained preparations with the possible exception of one sperm which was doubtfully inside.

4. Most of the selfed eggs produced abnormal embryos. Sperm were found inside the membrane in some eggs, but only a few in each case. The cross-fertilized eggs, to (3), gave 50 per cent normal tadpoles.

5. No cleavage or embryos were recorded, but the best preparations show a few sperms inside the membrane. No crosses were made.

Discussion

The preceding evidence shows very clearly that in most cases no sperms are found inside the membrane of self-fertilized eggs in cases where no cleavage or embryos were observed. In the last case (5), however, a few sperm were found. Since self-fertilization does take place to a considerable extent in *Styela* it is to be expected that sperm may enter some of the eggs. Cleavage or development would be expected, in some cases at least, provided one of the sperm entered at or near the antipole. If this did not happen it is doubtful if sperm reaching the surface elsewhere would enter the egg, and it is also possible that when a sperm does enter the egg in the antipole region, the surface of the egg may then become resistant to sperm, present at another part of the egg, as is known to be the case in many other eggs.

This examination shows that after suitable staining, i.e., when the sperm are sharply marked off from the contents of the test cells, the sperm are not present or rarely present in or between the test cells after self-insemination. Whether some of the entering sperm pass through or around the test cells is unimportant for the present discus-

sion. I have observed cases where the sperms that were inside the membrane were not inside the test cells, other cases where they appeared to lie on the surface of the test cells, and a few cases where they seemed to be inside these cells. The test cells of *Styela* do not completely surround the egg, as they do in *Ciona*. These cells are often grouped in twos, threes, or more with intervening spaces. Sperm entering these spaces could pass directly to the surface of the egg. There is not the slightest evidence, even after cross-fertilization, that the test cells are filled with sperm. Without suitable staining and extraction the threads and granules might easily be misinterpreted as spermatozoa.

Styela Montereyensis

To date I have not been able to bring about spawning in this species, but abundance of eggs and sperm can be obtained from large individuals by cutting open the animal, removing the digestive tract (including the pharynx), slicing off the ovaries and their ducts, and, necessarily, the testes also and sperm ducts at the same time. A cloudy suspension is obtained, the debris is removed, the eggs concentrated by revolving the dish, the supernatant fluid poured off, or else the eggs removed to sea water. Neither cross- nor self-fertilization takes place (in January) in many cases, but at times cross- and even some self-fertilization occurs, and these are the only ones in the following account that were used to test the entrance, or failure of the sperm to enter. The eggs were killed in Bouin's solution in most cases, cut into sections, and studied under an immersion lens. Active sperm are rarely observed, whether because of the presence of mucus from the body tissues, or because the spermatozoa are sluggish in this species, or are immature. The failure to cross-fertilize seems to be due to failure of the sperm rather than of the eggs. Attempts to activate the sperm by treatment with ether, or with various salts, have failed as a rule, but in a few cases by the use of lemon juice (2.7 pH) self-fertilization took place when it did not occur in eggs fertilized in sea water or had occurred to a smaller extent. A good many preparations of the eggs of this species were examined, both in cases where no cleavage or development took place, and in cases where crossing led to a considerable amount of cleavage and sometimes 100 per cent of embryos. In the former cases no sperm were found inside the membrane. The test cells in this species, as in the other *Styela*, contain yellow-red granules and threads of material that stain, but after proper extraction these lose their color while it remains in the heads of the spermatozoa. The latter stand out conspicuously, being stained deep red (acetic orcein). The sperms here, as in the other species, are large

and can be identified by their shape as well as their color. Nevertheless, I have not found any eggs, even in those that gave a high percentage of cleavage, with sperms inside the membrane, although one sperm at least must have entered at the antipole in the eggs that cleaved. The paucity of active sperms may in this case be the explanation of failure to find any inside the membrane.

Removal of the Test Cells from Most of the Egg's Surface

In order to find out whether the presence of the test cells plays a role in inhibiting the entrance of spermatozoa into their own eggs I centrifuged (1938) the unfertilized eggs of *Ciona* for two hours at a high speed (about 3,500 r.p.m.). The test cells were driven to one pole of the egg where they form a thick mass, or a zone of clear material. The surface of the rest of the egg is brought into contact with the membrane. Such eggs were tested by selfing and crossing. It was found that they do not self but will cross. Control normal eggs were tested in each set and gave corresponding results.

The number of experiments of this kind was small and I repeated the procedure in the autumn of 1941. The first test was negative, the eggs neither selfed nor crossed. I noticed, however, that the centrifuge that I used was quite warm after two hours, and failure of the eggs to cross-fertilize was undoubtedly due to the high temperature. To avoid this I removed the machine to a cold room (4° C.), but after two hours the test cells were not moved. The low temperature had, it appears, stiffened them or produced some other effect such that they were not moved. Then a different make of centrifuge was used that did not warm up during the two hours of rotation. The results in every case were uniformly good; the selfed eggs did not segment while practically all of the crossed eggs segmented.

The results confirm the earlier ones and demonstrate that the test cells are not concerned with the block to self-fertilization. The results do not show, of course, that the block may be in the surface of the egg. Other observations or methods are necessary to demonstrate that it is the membrane around the egg and not its surface that inhibits self-fertilization. Evidence bearing on this point was obtained some years ago (1923) by cutting the membrane which sometimes allows the whole egg either to squeeze out, or to pinch off fragments of different sizes. These exposed eggs and fragments self-fertilized and segmented. It might be argued that the operation itself had affected the surface of the egg so that it lost its power to block the entrance of its own sperm, but this seems to me a rather far-fetched argument, purely theoretical, since such exposed eggs gave normal cleavage and sometimes normal embryos.

SUMMARY

A comparison of unfertilized eggs of *Ciona* with self- and cross-fertilized eggs show that spermatozoa do not, as a rule, penetrate the membrane of their own eggs. Rarely a sperm may be found inside a self-inseminated egg, which is consistent with the fact that occasionally self-fertilization occurs. Even in cross-fertilized eggs only a very few sperm are found inside the membrane of such eggs. The same statement holds for two species of *Styela* examined, except that a few more sperm are likely to be found inside the membrane of the self-inseminated eggs, which is consistent with the fact that self-fertilization is more frequent in these species than in *Ciona*.

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BEHAVIOR OF THE NURSE-CELLS OF LITTORINA IRRORATA (SAY)

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As the writer has shown (in press) the mode of development of the nurse-cells of *Littorina irrorata* is in many respects such as to indicate their homology to the atypical spermatozoa of other prosobranchs. The present paper is concerned with the behavior and history of the nurse-cells since they furnish additional support to this view.

The methods used consisted simply in the examination of temporary mounts and permanent smears of the contents of the different parts of the reproductive tract of both sexes. This material was secured with a pipette during dissection of the animals in sea water. The smears were fixed and stained by the usual methods. These were useful as a check upon the observations made on living material, for photographs and as a permanent record of the observations.

Behavior of the Nurse-cells and Spermatozoa from the Sperm Duct

The general appearance of the nurse-cells with tufts of quiescent spermatozoa attached is shown in Figure 1. Such nurse cells were found abundantly in the first (coiled) portion of the sperm duct of every male examined (July). The seminal vesicle, prostate and the distal portions of the sperm duct were entirely empty in every case. When the nurse-cells are removed from the duct and examined in sea water all of the spermatozoa are at first quiescent. Within a few minutes, however, some of them begin to show slight movements of their tails. These movements gradually increase in amplitude, and more and more of the spermatozoa begin to move in the same way until the entire group attached to a given nurse-cell is beating rhythmically. The nurse-cell is thus propelled through the water until it reaches some obstacle or until the tails of the sperm of one tuft become entangled with those of another. The latter occurs in more and more tufts the longer the cul-

[1] For the use of the facilities of the Fisheries Laboratory the author is indebted to the Hon. John R. Gardner, Acting Commissioner of the Bureau of Fisheries. He also wishes to thank Dr. H. F. Prytherch, Director of the Beaufort Station, for many courtesies.

ture is maintained until finally (in about 30 minutes usually) hundreds of individual tufts have become entangled with each other. The nurse-cells themselves lie peripherally and are moved passively to and fro with the beating movements of the tufts. The three-dimensional pinwheels formed in the above manner (Fig. 4) often are seen to revolve in the liquid medium, if space permits. They may undergo progressive movement if there happen to be more sperm tufts on one side than on the other. The formation of these aggregates gives the culture a spotted appearance which is visible to the naked eye.

After some time a few of the spermatozoa of each aggregate become detached from the nurse-cells. Other spermatozoa become detached until finally all of the sperm attached to a given nurse-cell have lost contact with it. When this happens the nurse-cell set free in this way is forced peripherally by the currents set up by the moving spermatozoa. When all of the nurse-cells are free the spermatozoa still form an aggregate being completely entangled by their tails. These latter aggregates persist for some time. The heads of the spermatozoa are radially disposed around the periphery of the aggregate. After a time the spermatozoa become disentangled from the aggregate and swim freely away from it. The free spermatozoa show a marked thigmotaxis and soon become attached by their heads to any available surface but not to the free nurse-cells.

Behavior of Nurse-cells in Female

Some 200 females were dissected in order to follow the history and fate of the nurse-cells and spermatozoa. In only a small percentage of cases was a recent copulate to be found. The great majority of the females were found with empty uteri, but nearly all of them contained a large number of spermatozoa in the seminal receptacle. This organ is

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PLATE I

FIG. 1. Smear from sperm duct showing nurse-cells with attached spermatozoa. Osmic vapor. × 579.

FIG. 2. Smear from uterus showing nurse-cells with contained spermatozoa. A few free spermatozoa are to be seen here and there. Zenker's fluid without acetic. × 579.

FIG. 3. Proximal end of spermatophore taken from uterus showing the spermatozoa agglutinated head-to-head after segregation from nurse-cells. Osmic vapor. × 48.

FIG. 4. Three-dimensional pinwheel aggregate formed of many nurse-cells with attached sperm tufts. Somewhat distorted prior to fixation. Osmic vapor. × 48.

FIG. 5. Distal end of same spermatophore shown in Figure 3 showing the nurse-cells segregated from spermatozoa and about to be extruded. × 48.

FIG. 6. Section through female at the level of the seminal receptacle showing typical spermatozoa attached by their heads to the lining cells. × 5.6.

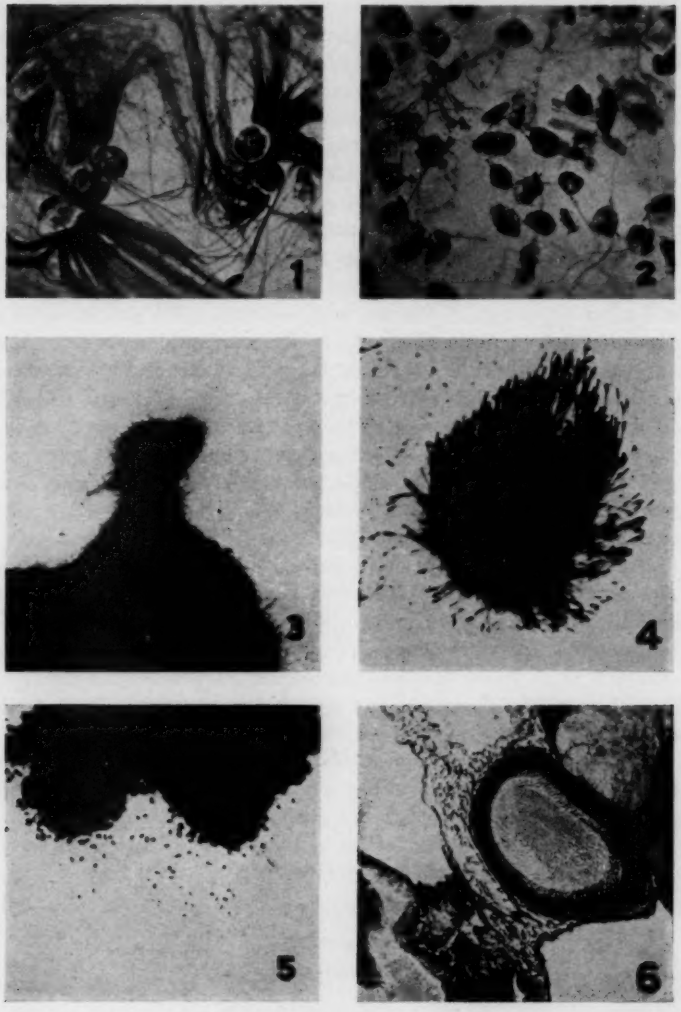

PLATE I

Figures 1 and 2 were made with a 4 mm., apochromatic objective (N.A. 0.95), Figures 3, 4 and 5 with a 16 mm., apochromatic objective (N.A. 0.30), and Figure 6 with a 32 mm., microtessar. The magnification of each figure is given in the descriptive legend.

a blindly ending sac lying posterior to the site of sperm deposition. Figure 6 shows a section through a female at the level of the seminal receptacle. The heads of the spermatozoa are attached to the epithelial cells lining the receptacle, the tails projecting into the lumen. No nurse-cells were found in the seminal receptacle. Linke (1933) found the same arrangement in the species which he studied, and he also observed sections of *L. obtusata* which included an egg as it passed the opening of the seminal receptacle into the oviduct. The egg was surrounded by spermatozoa which appeared to be streaming from the seminal receptacle, indicating that this is the site of fertilization.

In three of the females examined, the distal end of the uterus contained a cylindrical mass of semen which showed considerable resistance to breakdown when removed for study. In one case this mass contained a large number of nurse-cells with attached spermatozoa, in another case it consisted of nurse-cells and free spermatozoa, and in a third of nurse-cells only. Many of the nurse-cells in the latter copulate were swollen or otherwise distorted. In the second case cited above the nurse-cells were located at the distal end of the mass only, the spermatozoa being segregated at the opposite or proximal end, i.e., nearer the seminal receptacle (see Figs. 5 and 3, respectively).

In two of the females in which the above described sperm mass was not present the uterus contained, instead, a large number of swollen nurse-cells and a few spermatozoa. The nurse-cells were in the act of ingesting living spermatozoa, and many of them contained hundreds of sperm (Fig. 2). In many cases spermatozoa could be seen within these cells, coiling and writhing about in a large watery vacuole contained in the nurse-cell.

The spermatozoa of *L. irrorata* show a pronounced head-to-head agglutination reaction when not attached to nurse-cells. This is true of segregated sperm taken from the seminal receptacle as well as of those taken from the proximal end of a recent copulate. This agglutination can be seen in Figure 3 which represents the proximal end of such a copulate.

Discussion

The most remarkable aspect of the history of the nurse-cells lies in their behavior when withdrawn from the sperm duct. The formation of the three-dimensional pinwheels is similar in many respects to the formation of clumps of apyrene and eupyrene spermatozoa described by the writer (Woodard, 1940) for *Goniobasis:* 1. Both are brought about by the sticking together or entanglement of spermatozoa by their tails. 2. Both result in the formation of temporary aggregates. 3. In both

cases the atypical components play out their role and from this time on show no further ordered behavior towards the spermatozoa. 4. In *Goniobasis* a tail-to-tail aggregation of the spermatozoa is a condition intermediate to their final disposition (in the seminal receptacle). This is presumably also the case in *L. irrorata.* 5. In both cases these aggregates eventually disperse, and the free spermatozoa show a tendency to wander away from the site of aggregation, and a marked thigmotaxis. 6. In both cases the spermatozoa eventually become sharply segregated from the atypical structures and reach a seminal receptacle where they are stored. 7. In both cases the atypical structures undergo breakdown and extrusion after their functional phase has passed. 8. Finally, in both cases the atypical structures act as an aid in the aggregation. In the case of *Goniobasis* as I have shown experimentally, the atypical spermatozoa are necessary. In the case of *L. irrorata,* it may be emphasized, both nurse-cells and spermatozoa may be present separately, but when not attached the spermatozoa aggregate head-to-head (agglutinate) rather than by their tails. This would seem to indicate that an atypical structure is necessary for tail-to-tail aggregation.

The observations of Tyler (1940) may be of great significance in this connection. He has shown that in *Megathura crenulata* two types of sperm aggregation occur: one is a head-to-head aggregation which he regards as primarily a dilution effect; the other is a tail-to-tail agglutination which is effected by a substance present in the jelly layer of the egg. The existence of such a specific tail-to-tail agglutinin suggests that, in the case of both the apyrene spermatozoa of *Goniobasis* and the nurse-cells of *Littorina,* we may be dealing with structures which can in some way produce the same effect upon the spermatozoa as the substance from the jelly layer of the egg in Tyler's experiments. It may thus be possible to arrive at a biochemical proof of the essentially egg-like character of the atypical spermatozoa of prosobranchs where the morphological proof remains more or less indecisive.

Summary

The nurse-cells of *L. irrorata* with tufts of sperm attached are moulded in the male into rudimentary spermatophores and are then passed to the female. In the uterus the sperm become detached from the nurse-cells and migrate to the seminal receptacle where they are stored. The nurse-cells then ingest any remaining spermatozoa, break them down, and are themselves eventually extruded. Nurse-cells with tufts of sperm in the sperm duct show an aggregation reaction which leads to the formation of three-dimensional pinwheels. The tufts of

sperm are attached by their tails to each other, and the nurse-cells lie radially disposed. These aggregates disperse first by detachment of the nurse-cells, leaving the sperm attached by their tails, then by disentanglement of each of the sperm. The free sperm then show a thigmotaxis. Sperm not attached to nurse-cells (from seminal receptacle or from a copulate in which segregation has already occurred) show the usual head-to-head agglutination reaction. The behavior of the nurse-cells and spermatozoa is viewed as a mechanism whereby the latter are segregated from the former. This behavior is compared with the clumping of the apyrene and eupyrene spermatozoa of *Goniobasis*.

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